

NOVEL AFFINITY TECHNIQUES
by
MATTS RAMSTORP



**DEPARTMENT OF PURE AND
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NOVEL AFFINITY TECHNIQUES

av

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Abstract Some novel developments where affinity interactions are used for purification as well as analysis are presented and discussed. Cells of three different strains of streptococci and <i>Staphylococcus aureus</i> were immobilized by entrapment in polyacrylamide, packed in columns and used as affinity adsorbents for the binding of human serum proteins. The same principle was utilized for affinity purification of a substance in carrot (<i>Daucus carota</i>), capable of agglutinating cells of the oral microorganism <i>Streptococcus mutans</i>. Further studies on this substance revealed it to be a carbohydrate polymer of unknown composition, presumably a dextran. Partition Affinity Ligand Assay (PALA) was used for the quantitation of bacterial cells. The principle of this assay is that free and bound labelled antibodies are partitioned to different phases in an aqueous two phase system. Both <i>Staphylococcus aureus</i> cells and <i>Streptococcus B₁</i> cells were analyzed, and in the latter case the detection limit was 2 500 cells. The combination of ultrafiltration technology and affinity interactions, yielding a system where yeast cells were confined by hollow fibers to a recirculation stream, was used for purification of the lectin Concanavalin A. The product was shown by electrophoresis to be homogeneous and the yield was 70 %.			
Key words Affinity interactions, immobilized cells, aqueous two phase systems, Partition Affinity Ligand Assay (PALA), ultrafiltration, Ultrafiltration Affinity Purification, Enzyme Linked Immunosorbent Assay (ELISA)			
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APPLIED BIOCHEMISTRY**
Lund 1983

JOHN ALBERT THOMAS JR.
M.A. B.A. LL.B.



DEPARTMENT OF LAW AND
APPLIED ECONOMICS
LAW 100

To Pia and Anna

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LIST OF PAPERS

This thesis are based on the following papers, which are referred to by Roman numerals in the text.

- I. Affinity Chromatography Using Immobilized Bacterial Cells with Receptors for Human Serum Proteins.
B. Mattiasson, M. Ramstorp, K. Widebäck and G. Kronvall
J. Appl. Biochem., 2, 321-335 (1980)
- II. Isolation and Partial Characterization of a Substance from Carrots (*Daucus carota*), with Ability to Agglutinate Cells of *Streptococcus mutans*.
M. Ramstorp, P. Carlsson, D. Bratthall and B. Mattiasson
Caries Res., 16, 423-427 (1982)
- III. Applications of Partition Affinity Ligand Assay (PALA) in a Quick Test for Quantitation of *Staphylococcus aureus* Bacterial Cells.
B. Mattiasson, M. Ramstorp and T.G.I. Ling
J. Immunol. Methods, 41, 105-114 (1981)
- IV. Immunological Quantitation of Bacterial Cells Using a Partition Affinity Ligand Assay: A Model Study on the Quantitation of Streptococci B.
T.G.I. Ling, M. Ramstorp and B. Mattiasson.
Anal. Biochem., 122, 26-32 (1982)
- V. Ultrafiltration Affinity Purification. Isolation of Concanavalin A from Seeds of *Canavalia ensiformis*.
B. Mattiasson and M. Ramstorp.
Submitted.

INTRODUCTION

Developments in biochemistry, as well as in other biosciences, have to a large extent followed the development of purification and analytical techniques. Purifications of individual substances from complex biological mixtures are generally performed by combining several techniques in order to sort out the various components according to differences in their chemical and physical properties, e.g. charge, size and solubility. To obtain successful purifications, it is often necessary to combine different techniques so that complex mixtures will be successively fractionated.

It is generally found, when devising purification schemes, that chromatographic methods are employed in one or more steps. Chromatography is a separation technique whereby components in a mixture are separated with the use of a stationary phase through which a mobile phase, a gas or liquid, is forced. Some examples include: **Partition chromatography**, which separates molecules according to differences in solubility between the stationary and the mobile phase, respectively; **Gel filtration**, also called molecular sieve chromatography, which separates molecules according to molecular weight (size); and **Adsorption chromatography**, which is based on intramolecular interactions.

Affinity chromatography is a technique based on specific, reversible interactions between biologically complementary molecules, e.g. enzyme-inhibitor and antibody-antigen. Thus, affinity chromatography represents the ultimate form of adsorption chromatography.

The ligate molecule to be purified binds to the complementary ligand molecule, which has been immobilized to support material, called the matrix.

The first report on the use of affinity interactions for purification purposes was published in 1910, where α -amylase was purified by adsorption to insolubilized starch (Starkenstein, 1910). This was followed, after nearly half a century, by the purification of antibodies by affinity chromatography on antigens immobilized to cellulose (Champbell *et al.*, 1951). Due to the shortage of suitable support material, as well as reliable immobilization methods, affinity chromatography was prevented from becoming generally established at this point. However, the discovery that molecules containing primary amino groups could be coupled to cyanogen bromide activated polysaccharide materials (Axén *et al.*, 1967) offered an opening for more general use of affinity chromatography.

Separation methods based on specific interactions have two major requirements. First, a biospecific ligand must be available, which retains its binding ability after immobilization to the matrix. Second, methods must be available for desorption of substances subsequently bound to the ligands, after elimination of non-interacting material. The principle of affinity chromatography is schematically illustrated in Figure 1. In Table 1 some of the biological systems are listed which are most frequently utilized in affinity chromatography.

As is demonstrated in Table 1, a vast number of biological compounds have the capacity of specific recognition due to different types of interactions, including van der Waals forces, electrostatic and hydrophobic interactions. The interactions between a ligand molecule (L) and a ligate molecule (S) can be described by the equation:



where L-S denotes the ligand-ligate complex, and k_1 and k_{-1} are association and dissociation rate constants, respectively. The ratio between the dissociation and association rate constants:

$$k_1/k_{-1} = K \quad (2)$$

is the equilibrium constant, which describes the strength of the interaction between the ligand and the ligate molecules. The equilibrium constant values depend on the ligand-ligate system. For example, the avidin-biotin interaction represents an extremely stable complex ($K = 10^{15}$) whereas lectin-carbohydrate systems represent more labile systems ($K = 10^{-4}$).

Many of the systems listed in Table 1 involve strictly specific interactions. However, more general (or group specific) interactions can be utilized for separating a mixture of related substances. This means that the same general adsorbent can be used to purify several substances without the requirement of a new adsorbent for each different substance to be purified. Although these general ligands can bind several related substances, the substances can often be individually eluted from the chromatography column by means of specific elution procedures. Some general ligands are listed, together with their specificities, in Table 2.

Affinity chromatography clearly offers several unique possibilities for the fractionation of complex biological mixtures, which in practical work is manifested in the following way:

Table 1. Some of the most frequently utilized biological systems in affinity chromatography.

Ligate	Ligand
Enzyme	Inhibitor, substrate analogue, inhibitor
Antibody	Antigen, including virus and cells
Lectin	Carbohydrates, glycoproteins, cells surface receptors
Nucleic acid	Complementary base, histone, nucleic acid polymerase, binding protein
Hormone	Receptor
Cell	Lectin

Table 2. Some general ligand molecules and their specificities.

Ligand	Specificity	References
Protein A	Fc-region on IgG	Hjelm <i>et al.</i> , 1972 Ey <i>et al.</i> , 1978 Chenais <i>et al.</i> , 1977
Lectins	Carbohydrate residues	Lanner <i>et al.</i> , 1978 Ghosh <i>et al.</i> , 1978
Poly(U)	mRNA, interferon	Shapiro and Schimke, 1975 De-Maeyer-Guignard <i>et al.</i> , 1978
Poly(A)	Viral RNA, mRNA binding proteins,	Yoga and Wimmer, 1975 Fukami and Itano, 1976
Cibacron blue	NAD ⁺ /NADP ⁺ -requiring proteins, interferon	Easterday and Easterday, 1974 Jankowski <i>et al.</i> , 1976
5'AMP	NAD ⁺ -dependent dehydrogenases, ATP-dependent kinases	Brodelius and Mosbach, 1973

- * The separation is straight forward, since the adsorbent is specific for the substance of interest.
- * The use of general ligands, together with specific elution techniques, extends the applicability of a given ligand, with preserved specificity.
- * Since specific interactions are used, rather than more overall physical-chemical properties, affinity chromatography is well suited for the isolation of substances in very low concentrations, such as some lectins (Ersson, 1980).
- * Affinity based interactions may also be utilized in other techniques, such as affinity electrophoresis (Bøg-Hansen, 1973) and affinity partitioning (Shanbhag and Johansson, 1974).

Affinity chromatography lends itself to scaling up as well as to automation (Janson, 1982). The main disadvantage of large scale affinity chromatography is the limited mechanical stability of the matrix materials which are commercially available. However, ways of circumventing these problems have been suggested. Automation of affinity chromatographic applications in industry, as well as in laboratory research has been demonstrated (Robinson *et al.*, 1974; van der Loo and Hamers, 1976).

In large scale applications, batch procedures are most commonly preferred. Here the adsorbent is mixed with the solution containing the molecules of interest, incubated for some hours, collected by filtration or centrifugation, carefully washed and finally desorbed. Batch procedures are also preferred when handling viscous solutions, or when the solution to be purified contains particles.

With few exceptions, ligands for biospecific adsorption are very complex. Their synthesis involves many steps and they are thus very expensive. The use of such ligands on a larger scale can only be justified for expensive enzymes and other proteins. Here the use of more general ligands can give more economically favourable systems (Mosbach *et al.*, 1971).

Another important area for the application of affinity interactions is analysis, both quantitative as well as qualitative. The specificity of the ligands allows for very selective procedures. Table 3 lists some analytical procedures utilizing affinity interactions.

The papers presented in this thesis concern themselves with some novel developments in the utilization of affinity interactions. The first two papers (paper I and II) deals with immobilization of bacterial cells by entrapment in polyacrylamide gels. Paper I is a model study based on the interaction of materials with the surface structures of the immobilized bacterial cells.

Table 3. Some analytical techniques utilizing affinity interactions

Analytical procedure	References
Radioimmunoassay	Yalow and Berson, 1959
Enzyme immunoassay	Engvall and Perlmann, 1971
Immuno-electrophoresis	Scheidegger, 1955
Affinity-electrophoresis	Bøg-Hansen, 1973
Immunodiffusion	Mancini <i>et al.</i> , 1963
Rocket-electrophoresis	Laurell, 1966
Immunoprecipitation	Goettsch and Kendall, 1935
Agglutination	Adler and Liu, 1971

Various *Streptococcus* strains and *Staphylococcus aureus* were immobilized and studied for the binding of human serum proteins. A component in carrot which is capable of agglutinating oral streptococci has been purified by affinity chromatography using immobilized *Streptococcus mutans* cells as an adsorbent (paper II). This paper also describes further attempts to characterize the purified agglutinating substance.

Quantitative analysis of bacterial cells by utilizing both pseudo-immunological, as well as immunological, interactions has been performed according to the principle of Partition Affinity Ligand Assay (PALA). Paper III describes a model system for the quantitation of *Staphylococcus aureus* cells, using the pseudo-immunological interaction between protein A on the cell surface and the Fc-parts of IgG-molecules. Paper IV describes the quantitative analysis of *Streptococcus* cells utilizing an immunological interaction between the cells and anti-*Streptococcus* antibodies.

A novel affinity purification system is introduced in paper V. By using the surface structures of heat killed yeast cells (*Saccharomyces cerevisiae*), confined by hollow fibers to a recirculation stream, the affinity between the lectin Concanavalin A from *Canavalia ensiformis* and the carbohydrate structures on the yeast cells was utilized as a model system for the purification of this lectin.

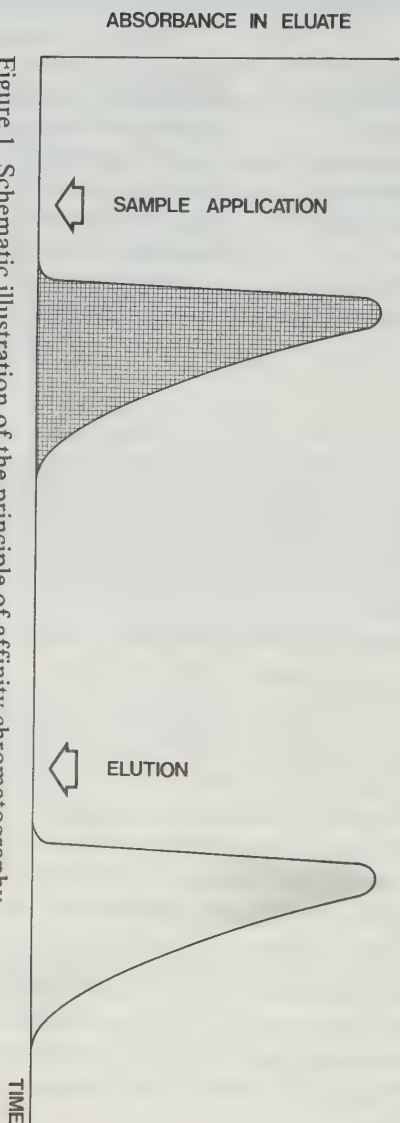


Figure 1. Schematic illustration of the principle of affinity chromatography.

A. The support with bound ligand, is packed in a column.

B. An extract, containing ligate molecules to be purified together with other molecules, is applied to the column. Ligate molecules binds to the ligands whereas the other molecules are washed out of the column.

C. Only ligate molecules now remains in the column.

D. The ligand-ligate complex is dissociated and ligate molecules are washed out from the column in a purified state.

IMMOBILIZATION TECHNIQUES

An integral part in the construction of affinity systems is the immobilization of various ligands. As discussed in connection with papers I - V, these ligands may consist of small molecules (such as inhibitors), macromolecules (such as enzymes) or even whole cells. It is therefore appropriate to give an overview of the various immobilization methods presently available.

Immobilized biological material can be defined as:

Biological material confined or localized to a certain defined region of space with retention of its properties, and which can be used repeatedly and continuously.

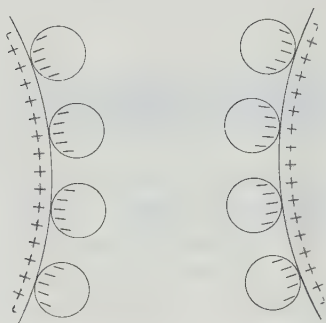
A wide variety of immobilization techniques have been developed during the past years. One possible way of categorizing these techniques is shown below, as well as illustrated in Figure 2.

- * Adsorption
- * Covalent attachment
- * Crosslinking
- * Encapsulation
- * Entrapment

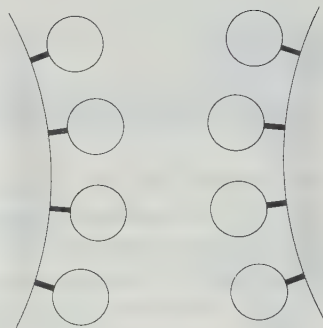
Depending on what is to be immobilized, the various techniques can be regarded as more or less suitable. In the following section the different techniques will be discussed with respect to their usefulness, giving special emphasis to cell immobilization. For more thorough information the following excellent review articles may be referred to (Abbot, 1977; Abbot, 1978; Chibata and Tosa, 1981; Birnbaum *et al.*, 1983).

Immobilization by adsorption

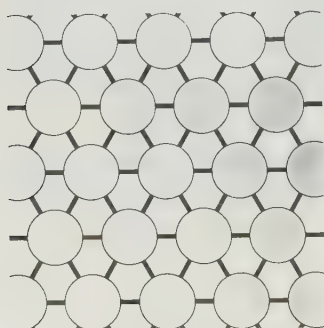
The advantage of adsorption lies in its simplicity. The material to be immobilized is mixed with the support material, yielding a very gentle immobilization procedure since the interaction is usually performed under physiological conditions. The procedure is also quick and the immobilized material



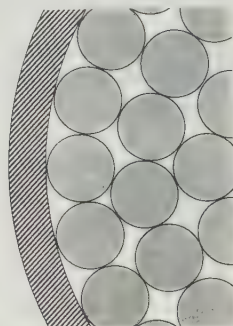
ADSORPTION



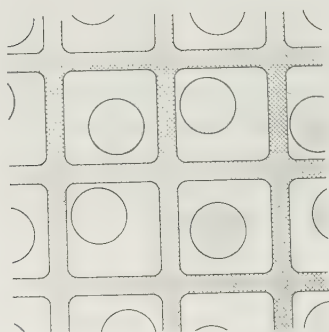
COVALENT ATTACHMENT



CROSSLINKING



ENCAPSULATION



ENTRAPMENT

Figure 2. A schematic illustration of the different immobilization techniques presently available.

can, in many cases, be eliminated at a later stage, thereby allowing reuse of the carrier material.

Immobilization by adsorption is dependent on several factors. These include the nature of the material to be immobilized, the composition, charge and shape of the carrier material, as well as the conditions under which the adsorption is performed.

The adsorption techniques, however, suffer from some disadvantages, the main one being leakage of immobilized material during use. This leakage is due to the fact that the bonds between the immobilized material and the support material are not too strong. Furthermore, in the case of cell immobilization, the cell concentration per unit volume will be limited due to the fact that the cells can only be adsorbed to the surface of the carrier material.

Nevertheless, adsorption techniques have proven useful in several applications, for example in the production of interferons using immobilized sensitive animal cells (Giard *et al.*, 1979). Adsorption of cells has also been used for the study of cell surface components, including antibody-antigen interactions (Wigzell and Andersson, 1969), lectin-carbohydrate interactions (Edelman *et al.*, 1971), as well as hormone-receptor interactions (Soderman *et al.*, 1973).

Immobilization by covalent attachment

An alternative technique to adsorption is covalent attachment of biological material to a support. As with the immobilized cell preparations obtained by adsorption techniques, covalently coupled cell preparations do not suffer from internal diffusion limitations since the cells are positioned on the surface of the carrier material. Immobilization via covalent bonds has, however, one great disadvantage. Due to the toxicity of the coupling reagents, the activity of the covalently coupled cells is generally lower than that of the corresponding free cells. Nevertheless, one way of overcoming this negative effect can be to activate the support material prior to the addition of the cells (Jack and Zajic, 1977).

Immobilization of cells by covalent attachment is of minor importance, especially if the preparations are to be used for production purposes. Reasons for this are:

- * Immobilization is usually not performed under sufficiently mild conditions.
- * Cell concentrations per unit volume are generally low.
- * High cost of coupling reagents.

Covalent coupling is thus not the first method of choice when immobilizing cells. It is, on the other hand, one of the most utilized methods for the immobilization of soluble biomolecules, such as ligands and enzymes. Table 4 contains some of the generally utilized activation methods suitable for covalent attachment of various biomolecules.

Table 4. Some common activation methods in immobilization by covalent attachment.

Activating reagent	References
Glutaraldehyde	Weston and Avrameas, 1971
Cyanogen bromide	Axén <i>et al.</i> , 1967
Hydrazine	Inman, 1974
Bis-epoxirane	Sundberg and Porath, 1974
Divinylsulfone	Fornstedt and Porath, 1975
Epichlorhydrin	Porath and Fornstedt, 1970
Benzoquinone	Brandt <i>et al.</i> , 1975
Periodate	Sanderson and Wilson, 1971
Trichloro-s-triazine	Finlay and Troll, 1978
Tresyl chloride	Nilsson and Mosbach, 1981

Immobilization by crosslinking

Immobilization of biological material can also be obtained by chemical (covalent) and/or physical (ionic) crosslinkage. Immobilization of cells by chemical crosslinkage is defined as the covalent attachment of cells to one another via polyfunctional reagents, such as glutaraldehyde (Chibata *et al.*, 1974), diisocyanate (Chibata *et al.*, 1974) and diamines (Lartigue and Weetall, 1976). Some commercially available immobilized whole cell glucose isomerase preparations are prepared by glutaraldehyde crosslinking (Hemmingsen, 1979). As is the case with covalent attachment techniques, however, one disadvantage of the crosslinking techniques is the toxicity of the reagents used.

Physical crosslinking via flocculation and pelletization is possible through the strong mutual adherence forces between some microorganisms and various materials. Such immobilized cell preparations can be obtained by incubation of the cells with polyelectrolytes, such as chitosan and other polyamines (Lee and Long, 1974; Tsumura and Kasumi, 1976). The method is simple, gentle, as well as cheap. However, the cell preparations thus obtained show limited mechanical stability, even if high cell concentrations per unit volumes can be obtained. One other disadvantage arises when the primary use of the immobilized cell preparation is for the study of interactions with cell surface receptors. In this case there is a direct correlation between the degree of crosslinkage and the steric blocking of surface structures.

Immobilization by microencapsulation

Immobilization by microencapsulation is based on the principle of confining material to a certain volume with the use of a semipermeable membrane, through which only smaller molecules are freely permeable. One advantage of this technique is that very high cell concentrations per unit volume can be obtained. However, the permeability of smaller molecules through the surrounding membrane is limited. Microencapsulation of enzymes has been utilized as a model for an artificial cell, whereby the enzymes are in an "intracellular environment" (Chang, 1964).

Immobilization by entrapment

Immobilization by entrapment within polymeric networks is by far the most commonly utilized and most versatile technique for the preparation of immobilized cells. Some advantages of this technique are:

- * Entrapment offers reduced cell leakage, compared to adsorbed cells.
- * Entrapment procedures are generally gentle, as well as simple, to perform.
- * Entrapment generally results in high cell concentrations per unit volume.

The various entrapment techniques are:

- * Thermal gelation
- * Precipitation
- * Ionic gelation
- * Polycondensation
- * Polymerization

ENTRAPMENT BY THERMAL GELATION

Agarose, kappa-carrageenan, collagen and gelatin are some of the naturally occurring polymers that are water soluble and have the property of forming gels when cooled down or dried. This property makes these materials suitable for entrapment of biomaterials, but because of the poor mechanical, as well as chemical, stability of such preparations, these types of carriers are generally stabilized with hardening agents, such as glutaraldehyde, subsequent to gelation.

Agarose, which is obtained from certain marine algae, is normally used as a support material when culturing cells. The properties of agarose include biocompatibility and high stability towards microbial degradation. Immobilization in agarose is performed by mixing material with a warm agarose solution (generally around 50°C), followed by cooling of the suspension below the gelation temperature. The gel block thus formed is then subsequently granulated or cut into smaller particles of desired size. Alternatively, agarose beads containing entrapped biomaterial can be obtained by dripping a warm agarose-cell suspension into a cold organic phase (Toda and Shoda, 1975), or by pouring the agarose-cell suspension into a cold organic phase containing a detergent, kept under stirring (paper I).

Kappa-carrageenan is a non-toxic, water soluble, highly charged polysaccharide which is obtained from various species of red sea weeds. It has found wide use as a food additive and has also proven to be useful and suitable for the entrapment of cell due to the fact that it forms gels under mild conditions (Takata *et al.*, 1977; Tosa *et al.*, 1979). Kappa-carrageenan solidifies upon cooling, but requires the continuous presence of cations, such as K^+ , Ca^{2+} or NH_4^+ , in order to maintain the gel state, unless it is treated with hardening agents (Takata *et al.*, 1977).

Immobilization in kappa-carrageenan is performed by mixing a warm solution of carrageenan with a cell suspension, after which the mixture is cooled down or contacted with gelling agents (Takata *et al.*, 1977). Alternatively, gel beads can be obtained by dripping the warm solution into an aqueous solution containing an appropriate cation (Tosa *et al.*, 1979).

Collagens, the major constituents of connective tissues, are proteins that are soluble at acidic pH (around pH 3 - 4) with the property of precipitating when the pH is raised to neutral or alkaline levels. This gelling property has been utilized for the entrapment of cells. Several immobilized cell systems have been studied to date, including bacteria, fungi and algae (Vieth and Venkatasubramanian, 1978).

Immobilization in collagen involves mixing the cell suspension with a collagen dispersion and then raising the pH into the alkaline region. The dispersion is then cast as a thin film, dried and finally treated with stabilizing agents, such as glutaraldehyde. The use of this technique for cell immobilization is limited by the fact that extremes of pH and temperature are used, which in many cases can be destructive to the cells.

Gelatin, a refined form of collagen, has also been utilized for the immobilization of cells (Nilsson and Mosbach, 1980).

ENTRAPMENT BY PRECIPITATION

Cellulose (Linko *et al.*, 1980), polystyrene (Hackel *et al.*, 1975) and EudragitTM (Klein and Wagner, 1980) are some polymeric materials which undergo precipitation, thus forming insoluble carriers into which cells can be immobilized. The immobilization of cells in cellulose, for example, is performed by mixing the cells with cellulose previously dissolved in an organic phase. This cell suspension is precipitated when placed in a water phase. In order to improve the stability of the preparation, and thereby also reduce the cell leakage, the gel beads are often treated with hardening agents, such as glutaraldehyde. Since they are exposed to organic solvents, the immobilized cells are generally obtained in a non-viable state, unable to carry out more complex reactions.

ENTRAPMENT BY IONIC GELATION

Salts of alginic acid, prepared from algae, are normally used as conditioning agents in food and as clarifying agents in fruit juices and beverages. Alginate is a linear polysaccharide which gels when in contact with multivalent counter ions.

Soluble sodium alginate is dissolved in water and carefully mixed with the cells to be immobilized. When this mixture is brought into contact with multivalent ions, such as Ca^{2+} , Al^{3+} or Fe^{3+} , a solid gel is formed in which

the cells are entrapped. The structure and size of the obtained gel is dependent on which type and concentration of alginate is used, as well as the gel forming procedure itself (Hackel *et al.*, 1975).

When forcing the alginate-cell suspension through a hypodermic needle, droplets are formed which solidify immediately into spherical beads when allowed to fall into a solution of multivalent counter ions, such as Ca^{2+} or Al^{3+} . Before the beads are used, they are first allowed to incubate for a couple of hours in the counter ion solution in order to increase the mechanical stability of the beads. To maintain stable gels during use, and at the same time reduce the leakage of cells, the counter ions must be continuously present in suitable concentrations. Gel stability can also be improved by cross-linkage of the alginate network, which has been reported to largely eliminate the need of counter ions in the system during use (Birnbaum *et al.*, 1981).

Gels made from alginate are generally highly porous with only small fractions of the bead being inaccessible. The immobilization of cells in alginate is, furthermore, a reversible process. This property may be of interest in some situations. By incubating the gel particles in solution of molecules capable complexing the counter ions, the particles will dissolve. Care must be taken, when designing an operational system, in order to avoid unwanted break down of the gel particles due to dissolution.

ENTRAPMENT BY POLYCONDENSATION

Polycondensation of epoxy- (Klein and Eng, 1979) or isocyanate- (Tanaka *et al.*, 1979) substituted polymers can be used to obtain immobilized cell preparations. Such preparations often have desirable support properties and the cells generally have good activities after immobilization, even though the reagents used are toxic to the cells.

ENTRAPMENT BY POLYMERIZATION

The most frequently used method for the immobilization of cells via entrapment techniques is to include the cells in polyacrylamide and related networks (Mosbach and Mosbach, 1966). The cells are entrapped by mixing the cell suspension with a solution containing monomers and crosslinkers. The polymerization process is started by the addition of a catalyst system. After block polymerization, the gel is subsequently granulated or cut into smaller particles of desired size (Larsson and Mosbach, 1976; Larsson *et al.*, 1979).

Alternatively, gel beads can be obtained by suspension polymerization in an organic phase, containing an emulsifying agent (Nilsson *et al.*, 1972; paper I).

Polymerization of acrylamide and acrylamide derivatives can also be initiated by illumination (Fukui *et al.*, 1978; Omata *et al.*, 1979) or by irradiation (Kaetsu *et al.*, 1979).

Other immobilization methods

Other methods for immobilization of biological material have also been described in the literature. A very simple and gentle method is to confine the material of interest to a certain defined space with the use of filters and membranes. For example, hollow fiber confined cells have been used for the production of urocanoic acid (Kan and Shuler, 1978), for denitrification of water (Mattiasson *et al.*, 1981), as well as for purification of material interacting with cell surface structures (paper V). Membranes have also been used to regenerate coenzymes (Wichman *et al.*, 1981) and in the analytical field in order to keep the cells immobilized at the surface of an electrode (Kobos *et al.*, 1979; Neujahr and Kjellén, 1979).

Another technique related to immobilization is the partitioning of biomolecules and cells to one phase of an aqueous two phase system. Aqueous polymer phase systems have been utilized for the production of ethanol from glucose using yeast cells (Kühn, 1980), for conversion of cellulose into ethanol (Hahn-Hägerdal *et al.*, 1981) and for the separation of free from bound antibodies in immunological quantitation of bacterial cells (paper III and IV).

AFFINITY CHROMATOGRAPHY ON IMMOBILIZED BACTERIAL CELLS

Various different techniques for the immobilization of microbial cells have been studied (paper I) with respect to the possibility of purifying molecules interacting with structures on the surface of the immobilized cells. Such affinity chromatography-like interactions have been studied with *Staphylococcus aureus* cells, together with three different streptococci strains which show different binding patterns for human serum proteins.

Protein A

Bacterial cells have been shown to be able to bind several host-derived proteins. The reason for this type of interaction has long been disputed and several alternative suggestions have been proposed, for instance that the invading microorganism can thus protect itself from the host's defence system.

In 1959 it was demonstrated that extracts of 75 % of all tested strains of *Staphylococcus aureus* were able to precipitate normal human serum (Jensen, 1959). Jensen claimed that this reaction was due to a cell wall polysaccharide which he called antigen A. Antigen A was later established to be a protein (Löfkvist and Sjöquist, 1962) and was given the name protein A (Grov *et al.*, 1964). The first interpretation for the precipitation reaction between protein A and immunoglobulin was that it represented a naturally occurring human anti-*Staphylococcus* antibody interaction, but this was later shown not to be the case. Forsgren and Sjöquist reported the reaction to be due to interactions between protein A and the Fc-H-chain of IgG (Forsgren and Sjöquist, 1966). Furthermore, they called this reaction "pseudo-immunological". Further studies revealed that IgG, originating from various species shows protein A reactivity (Sjöquist *et al.*, 1967; Forsgren and Sjöquist, 1967; Kronvall, 1973). However, only human subclasses IgG(I), IgG(II) and IgG(IV) are able to interact with protein A (Kronvall and Williams, 1969). This fact has been utilized for the purification of human IgG(III) (Hjelm, 1975).

Various streptococci strains also show Fc-reactivity similar to that of protein A (Myhre and Kronvall, 1980). Furthermore, several other serum proteins can interact with surface structures on bacterial cells.

Immobilization methods studied

The various methods studied include covalent attachment of cells to Sepharose, adsorption to IgG coupled to Sepharose, followed by crosslinking, and various entrapment methods using agarose, alginate, collagen and polyacrylamide. The results from these investigations are shown in Table 5. It was concluded that entrapment methods are to be preferred, entrapment of cells in polyacrylamide being an especially good choice.

When producing gel beads containing entrapped cells for use in column applications, some general properties are desirable:

- * The beads should be small enough to reduce diffusion limitations.
- * The beads should be large enough to prevent high pressure drops in the columns that may lead to compacting of the gel bead.

In an attempt to fulfill both of the requirements, a modified suspension polymerization was developed. This method resulted in larger clusters containing a large number of smaller beads.

This type of beads was obtained by mixing the cells with a solution containing monomers and crosslinkers. A catalyst system was then added and this hydrophilic phase was rapidly transferred into a heavily stirred hydrophobic phase containing a surfactant. The cell-containing phase formed small droplets in the hydrophobic phase and polymerized into small beads. When the small beads became visible, the stirring speed of the hydrophobic phase was decreased, thus allowing the small beads to aggregate into larger clusters. Figure 3 shows a micrograph of a cluster obtained by using this polymerization process.

Both mixtures of purified albumin and IgG, as well as pooled human serum, were added to the various column materials. The eluted and dialyzed material was analyzed with rocket immunoelectrophoresis using anti-albumin and anti-IgG antibodies, respectively.

The results obtained were in line with previously reported findings (Kronvall *et al.*, 1979; Myhre and Kronvall, 1980). However, in one case (namely *Streptococcus* G148) a peak in the 280 nm absorbance was observed that upon further analysis could not be identified.

Table 5. Evaluation of immobilization procedures tested with *Staphylococcus aureus* and streptococci cells.

Carrier material	Immobilization procedure	Results
Sepharose	CNBr-activation	Low yield
IgG-Sepharose	Adsorption followed by crosslinking	Low yield
Agarose	Bead formation in an organic phase	High yield Low stability
Alginate	Bead formation in a counter ion solution	High yield Low stability
Alginate-collagen	Bead formation in a counter ion solution alginate dissolved	Low yield Low stability
Crosslinked poly-acrylamide	Bead formation in an organic phase	High yield High stability

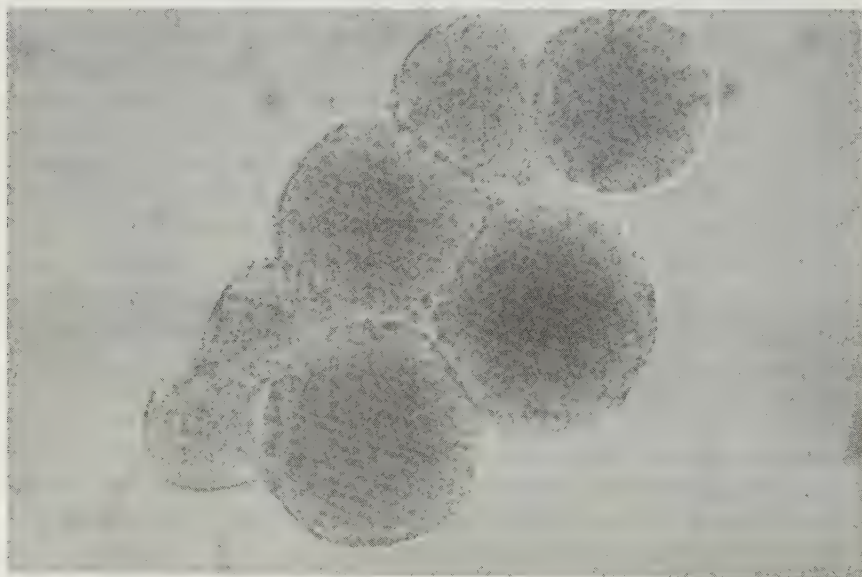


Figure 3. A micrograph of a cluster of beads containing immobilized bacterial cells.

Streptococcus mutans cell agglutinating substance

Although not all people are affected equally, tooth decay is a world-wide problem. Disintegration of the structure of the tooth, beginning at the surface and progressing inwards, results in dental caries. First, the surface enamel, which is entirely non-cellular, is demineralized. This has been attributed to the effect of bacterial acid products. Subsequent decomposition of the dentrin and cement involves bacterial digestion of the protein matrix.

An essential first step in caries production appears to be the formation of plaque on the hard, smooth enamel surface. The plaque consists mainly of gelatinous deposits of high molecular weight carbohydrate polymers in which the acid producing bacteria are immobilized. The carbohydrate polymers are mainly produced by streptococci, such as *Streptococcus mutans*, *Streptococcus sanguis* and *Streptococcus salivarius*, perhaps in association with actinomycetes. The essential second step appears to be the formation of large amounts of acid products by the cells in the plaque, from the breakdown of carbohydrates. High concentration of acid demineralizes the adjoining enamel and initiates caries.

The adherence of cells to the smooth enamel surface requires both the synthesis of water insoluble carbohydrate polymers and the participation of binding sites on the surface of microbial cells. Dental decay in teeth can be summarized to be a result of interactions between the host tissue and extremely specific cariogenic microorganisms, which can utilize nutrients provided by the host's diet.

Saliva contains substances which are capable of agglutinating certain bacteria in a lectin-like fashion (Magnusson, 1976). Other lectin-like substance have been observed to agglutinated certain oral streptococci (Bratthall, 1978), i.e. a substance in carrots, *Daucus carota*. Paper II is concerned with the purification of the carrot substance responsible for the agglutination of *Streptococcus mutans* cells by affinity chromatography on immobilized oral streptococci. The purified substance was subsequently studied in order to examine this interaction in greater detail.

Cells of *Streptococcus mutans*, strain OMZ 65, were immobilized according to the principle discussed in paper I. A crude extract of carrot was applied to the column material and washed free from non-interacting substances. Material bound to the column was then eluted, collected, dialyzed and concentrated before being analyzed for agglutination activity towards *Streptococ-*

cus mutans cells. The fractions containing cell agglutinating material were pooled and studied further. Various treatments were performed on the cells and the agglutinin, respectively, after which the treated material was examined for agglutination. Cells treated with trypsin, heat and dextranase, respectively, were incubated with untreated purified agglutinin, Table 6. Agglutinating substance, treated according to the same scheme, was incubated with untreated cells, Table 7. From these results it was concluded that the agglutinating substance was of non-protein origin, whereas protein structures on the cell surface were essential for the agglutination process.

In order to further study the agglutinin from carrot, attempts were made to perform inhibition studies with dextrans. When incubating cells together with high molecular weight dextrans, agglutination occurred. When the same experiment was performed using low molecular weight dextrans, no agglutination occurred. This resulted in the possibility of performing an inhibition assay using cells and agglutinin together with low molecular weight dextrans. This examination resulted in no observable agglutination, indicating that the material purified from carrots is some sort of carbohydrate polymer, presumably a dextran.

Table 6. Effects on various treatments of *Streptococcus mutans* cells on the agglutination by purified carrot agglutinin.

Cells treated with	Trypsin	Heat	Dextranase	Control
Agglutination	—	—	+	+

Table 7. Effects on various treatments of purified carrot agglutinin on the agglutination of *Streptococcus mutans* cells.

Agglutinin treated with	Trypsin	Heat	Dextranase	Control
Agglutination	+	+	—	+

AQUEOUS PHASE SYSTEMS IN ANALYTICAL APPLICATIONS

Old and conventional laboratory methods are frequently employed when identifying and quantifying microorganisms (Rytel, 1979a). During the past years, however, more and more research in the field of analytical microbiology has been aimed towards the development of quick and sensitive microbial assays (Johnston and Newson, 1977; Rytel, 1979b). These new methods are based on the metabolisms of living cells, cell surface properties of intact cells, or even loose fragments of the cell surface. Cell metabolism can be studied by gas chromatography of metabolites (Phillips *et al.*, 1977; Coloe, 1977) or conductivity measurements (Kahn *et al.*, 1977). Cell surface properties can be studied by immunological quantitation of intact cells (Kronvall, 1973; Edwards and Larsson, 1974), as well as loose cell surface fragments (Pickering, 1976; Coonrod and Rytel, 1978). Among the great varieties of immunological methods available for quantitation of microorganisms, most are based on equilibrium conditions, when the binding of antibodies to the cells take place.

Papers III and IV are concerned with a new approach for cell quantitation. The principle of this method, Partition Affinity Ligand Assay (PALA), is to make use of the differences in the ability of the components in a binding assay to partition in an aqueous two phase system. The PALA method is normally performed as a non-equilibrium method, i.e. the binding reaction does not have to reach equilibrium. Furthermore, the method gives the possibility of quantitating whole cells, cell fragments, as well as soluble structures in one and the same assay.

Aqueous two phase systems

If aqueous solutions of some water soluble polymers are mixed with each other, the mixture will, after some time, separate into distinctly separated phases. This phenomenon has been known for a long time (Beijerneck, 1896; Albertsson, 1958) and occurs in mixtures of certain polymers or between certain polymers and salts, depending on the concentrations. Aqueous phase systems have been used in biochemical preparative work and in the

study of surface properties of cell populations. It is also known that molecules partition in different ways in such phase systems. These partition differences have been employed in the purification of a vast number of macromolecular substances, such as proteins and nucleic acids (Albertsson, 1971). It is also possible to separate cells according to their surface properties (Albertsson, 1977a). Some examples of cells that have been partitioned in aqueous polymer phase systems are algae (Walter *et al.*, 1970), bacteria (Magnusson and Johansson, 1977; Stendal *et al.*, 1973), Chloroplasts (Larsson *et al.*, 1971; Andersson *et al.*, 1976; Andersson and Åkerlund, 1978), Erythrocytes (Walter and Selby, 1966; Walter, 1977), HeLa cells (Pinaev *et al.*, 1976) and Yeast cells (Johansson, 1974).

Partitioning of cell organelles, as well as other cellular particles, has also been studied. Some examples include mitochondria (Erikson, 1974; Gardeström *et al.*, 1978), plasma membranes (Lesko *et al.*, 1973), ribosomes (Pestka *et al.*, 1976) and synaptic vesicles (Hartman and Heilbronn, 1978; Flanagan *et al.*, 1976).

PROPERTIES OF AQUEOUS PHASE SYSTEMS

When mixing sufficiently concentrated solutions of certain water soluble polymers, aqueous phase systems are obtained. In a two phase system, the top phase is rich in one of the polymers and the bottom phase is rich in the other polymer. Compared to the phase systems most commonly utilized in chemical separations, i.e. those immiscible systems containing, for instance, an organic phase and water, aqueous phase systems are better for the partitioning of biological material. Aqueous phase systems are mild, i.e. the water content can be as high as 95 %, and can also be made to resemble physiological conditions by the addition of various low molecular weight substances, such as sugars and salts. When using aqueous phase systems for the partitioning of complex and fragile structures, such as cells and cell organelles, no destructive effects are demonstrated. On the contrary, stabilization of the partitioned material is generally observed.

Soluble material is mainly distributed to both of the two bulk phase of the phase system, whereas partitioning of particles such as cells, often results in partitioning of the particles to one of the two bulk phase, as well as to the interface between the two bulk phases. The distribution of soluble molecules can be characterized by the partition coefficient, K_{part} , defined as the ratio of the concentrations in the top and bottom phase, respectively:

$$\frac{C(\text{top phase})}{C(\text{bottom phase})} = K_{\text{part}} \quad (3)$$

Many parameters influence the partitioning behavior of a substance. Of great importance are the properties of the phase constituents themselves and the characteristics of the substance to be partitioned.

A typical phase diagram for an aqueous two phase system has been schematically illustrated in Figure 4. The curved line in this diagram is called the binodal curve, which is the line representing the minimum polymer concentrations required to obtain two phases. Thus, when mixing polymer solutions with concentrations above the binodal curve, the result is phase separation, whereas mixing polymers with concentrations below the curved line results in homogeneous solutions.

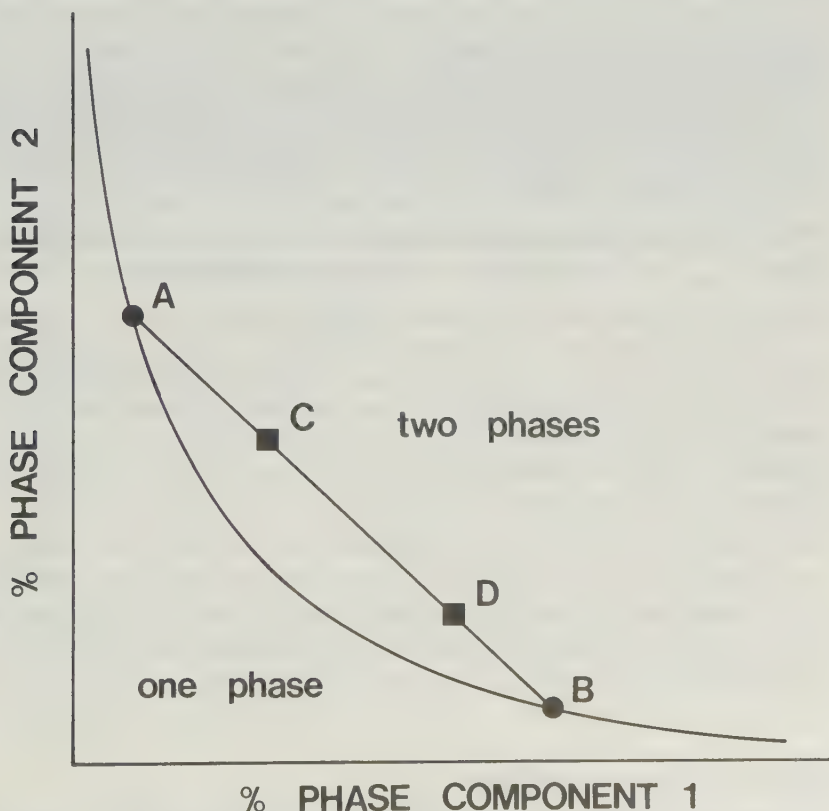


Figure 4. A schematic representation of a phase systems, consisting of two phase components 1 and 2, respectively. The curved line represents the border between homogeneous and two-phase system.

The time required for phase separation varies from phase system to phase system. In analytical applications there is a demand for quick separation, especially in the case of non-equilibrium assays such as the PALA-method. The phase systems employed in paper III and IV, have been chosen for their ability to rapidly separate. Two different phase systems have been employed, namely poly (ethylene glycol) (PEG) and magnesium sulfate (which is completely separated within 10 to 15 minutes), and PEG and dextran (which separates within 5 to 60 minutes). If only one of the phases is to be monitored for label, it is possible to obtain this phase much more rapidly in a pure state, before the other phase has clarified, by varying the ratio between the volumes of the two phases. This is because the volumetrically smaller phase is more rapidly clarified than the larger one.

Modification of partitioning

Biological macromolecules, cell organelles and cells are distributed in aqueous polymer phases system according to their surface properties. The partitioning of biological material can often be manipulated by modification of the phase system parameters.

When distributing biomolecules in aqueous phase system, the partition coefficient, K_{part} , can be divided into several contributing factors:

$$\ln K_{\text{part}} = \ln k_{\text{el}} + \ln k_{\text{hydrophob}} + \ln k_{\text{hydrophil}} + \ln k_{\text{conf}} + \dots \quad (4)$$

Where k_{el} , $k_{\text{hydrophob}}$, $k_{\text{hydrophil}}$ and k_{conf} are the partition coefficient factors due to electrical, hydrophobic, hydrophilic and conformational effects, respectively (Albertsson, 1977b). In the case of partitioning of particles, the situation is much more complicated due to the possibility of adsorption to the interface between the two bulk phases.

Since the partition coefficient can be divided into the above mentioned factors, there exist several possibilities for markedly increasing the separation effect by adding different low molecular weight substances, phase components with covalently attached molecules, as well as heavily modified "separator molecules".

Influence of salt on partitioning

To ensure a suitable environment for substance to be partitioned, various salts and sugars can be added to the phase system. The partition coefficient, K_{part} , for such low molecular weight substances is generally around 1, which

means that the molecules are evenly distributed between the two phases. However, some low molecular weight substances do not follow this pattern, which results in an uneven distribution of the molecules in question. If the ions of a salt are unevenly distributed in a phase system, an electrical potential will be built up between the phases. This phenomenon will, in some cases, drastically alter the partitioning of biological macromolecules and cells (Albertsson, 1965).

Influence of charged polymers on partitioning

Another alternative approach to drastically altering partitioning of biomolecules and cells in an aqueous two phase system is to add small amounts of phase polymers with covalently attached charged groups. Both negatively as well as positively charged polymers have been used. Positively charged polymers are obtained by substitution of the hydroxyl groups of PEG with trimethylamine, yielding trimethylamine poly (ethylene glycol) (TMA-PEG). Negatively charged polymers are obtained by the same substitution with sodium sulfite, resulting in the formation of poly (ethylene glycol) sulfonate (PEG-S) (Johansson, 1970). Since the charged groups are covalently coupled to PEG-molecules, these will now partition to the PEG-rich top phase of a PEG-dextran phase system, thus resulting in an uneven distribution of charges in the phase system.

The introduction of charged polymers often results in drastic changes in the partitioning behavior of biological material, even when using as little as 1 % charged versus uncharged polymer in the system. When using positively charged TMA-PEG, negatively charged biomaterial will be attracted to the PEG-rich top phase. On the other extreme, when using the negatively charged polymer, PEG-S, negatively charged biomaterial will partition to the opposite phase due to the repulsive forces of the charged groups in the PEG-rich phase. For positively charged biomaterial, the situation described above will be reversed.

Proteins can also be forced to partition according to their isoelectric points. This effect can be obtained by partitioning proteins in phase systems containing constant amounts of charged polymers and by varying only the pH of the system (Johansson *et al.*, 1973).

Influence of hydrophobicity on partitioning

If the electrical contribution, k_{el} , can be inhibited, it is possible to let other

factors determine partitioning behavior. The polymers used in aqueous polymer phase systems can be regarded to be more or less hydrophobic (Albertsson, 1971). In the phase system comprised of PEG and dextran, for instance, PEG is more hydrophobic than dextran.

The hydrophobic effects on partitioning can be made more pronounced by the addition of small amounts of polymers containing hydrophobic groups, obtained by covalent attachment of fatty acids to the polymers (Shanbhag and Axelsson, 1975). An alternative approach to changing the hydrophobicity of the phase system is to add small amounts of detergents. The detergent will thereby interact with hydrophobic portions of the material to be partitioned, resulting in a masking of the hydrophobicity (Johansson, 1976; Westrin *et al.*, 1976; Shanbhag and Axelsson, 1975; Johansson and Westrin, 1978).

Surface properties of cells have, for example, been studied by addition of polymers containing saturated, as well as unsaturated, fatty acids to polymer phase systems (Eriksson *et al.*, 1976). Similar systems have also been used in the study of liposomes (Eriksson and Albertsson, 1978). Soluble biomolecules have also been partitioned in such hydrophobic phase systems, but the effect is much more evident when partitioning cells and other particles. For example, red blood cells are partitioned from the bottom phase to the top phase by the addition of palmitoyl-PEG in concentrations as low as 0.0001 - 0.001 % (Eriksson *et al.*, 1976).

Influence of biospecific interactions on partitioning

The partition coefficient of a complex formed between two interacting molecules is not generally identical with that of either of the two molecules when these are partitioned alone. This fact can be used in the study of interactions between biomolecules (Backman and Johansson, 1976). Biospecific interactions similar to conventional affinity interactions have been employed for specific partitioning of biomolecules in aqueous phase systems. This method, called Affinity Partition, is based on biospecific interactions between two molecules, one of which has been coupled to one of the phase polymers (Shanbhag and Johansson, 1974; Flanagan *et al.*, 1976; Hubert *et al.*, 1976). The polymer containing the covalently attached ligand molecule partitions to the phase rich in the same unsubstituted polymer. Thus, the molecule specifically interacting with the ligand is thereby partitioned to the same phase, even if the interacting molecule normally partitions to the other phase of the system.

Partition Affinity Ligand Assay (PALA)

Aqueous polymer phase systems have, as mentioned before, been used for a long time in preparative biochemical work. Only recently have these phase systems been employed for analytical applications. As stated above, there is a demand that the reactants in an assay partition to different phases of the system. If this is not accomplished, the analysis can not be set up. In some cases the reactants partition spontaneously to different phases (Mattiasson and Eriksson, 1982; Mattiasson and Ling, 1980), but often they are obtained in one and the same phase. When the latter case is at hand, the desired partitioning can be obtained by modification of one of the reactants, i.e. by converting this reactant into a "separator molecule". The conversion is brought about by covalent attachment of a suitable polymer to the reactant in question.

The reactant which has been modified partitions to the phase rich in the polymer used for modification. The reactants of the assay are thereby partitioned into different phases and, furthermore, the interactions complex formed between the "separator molecule" and the other unmodified reactant will presumably show a preference for the phase to which one of the two reactants spontaneously partitions. When employing phase systems having differences in hydrophobicity between the two phases, such as PEG-dextran and PEG-magnesium sulfate systems, the "separator molecule" may either be made more hydrophobic or more hydrophilic. A more hydrophobic "separator molecule" will partition to the more hydrophobic PEG-phase, whereas a more hydrophilic "separator molecule" will partition to the more hydrophilic bottom phase.

The assay can be performed in two different ways, either as a direct binding assay, whereby the binding between two different molecules is employed to quantitate one of them, or as a competitive binding assay, which is based on a competitive situation whereby the labelled antibodies can bind to either modified cells or native cells, i.e. the cells to be quantitated.

In the direct binding assay, which is schematically illustrated in Figure 5, the labelled antibodies, *per se*, partition to the top phase of the two phase system. When performing an assay, the cells to be quantified are incubated with labelled antibodies, allowing interaction to occur, after which a well mixed phase system is added. The reaction mixture will thus separate into two phases according to Figure 5.c. After the phases have settled, a sample is taken from one of the phases and is analyzed for the presence of label.

This type of assay has the requirement that the two reactants must be collected in separate phases of the phase system. Normally this is, however, not the

case, but in practise it is sufficient if only one of the reactants has a distinct partitioning pattern.

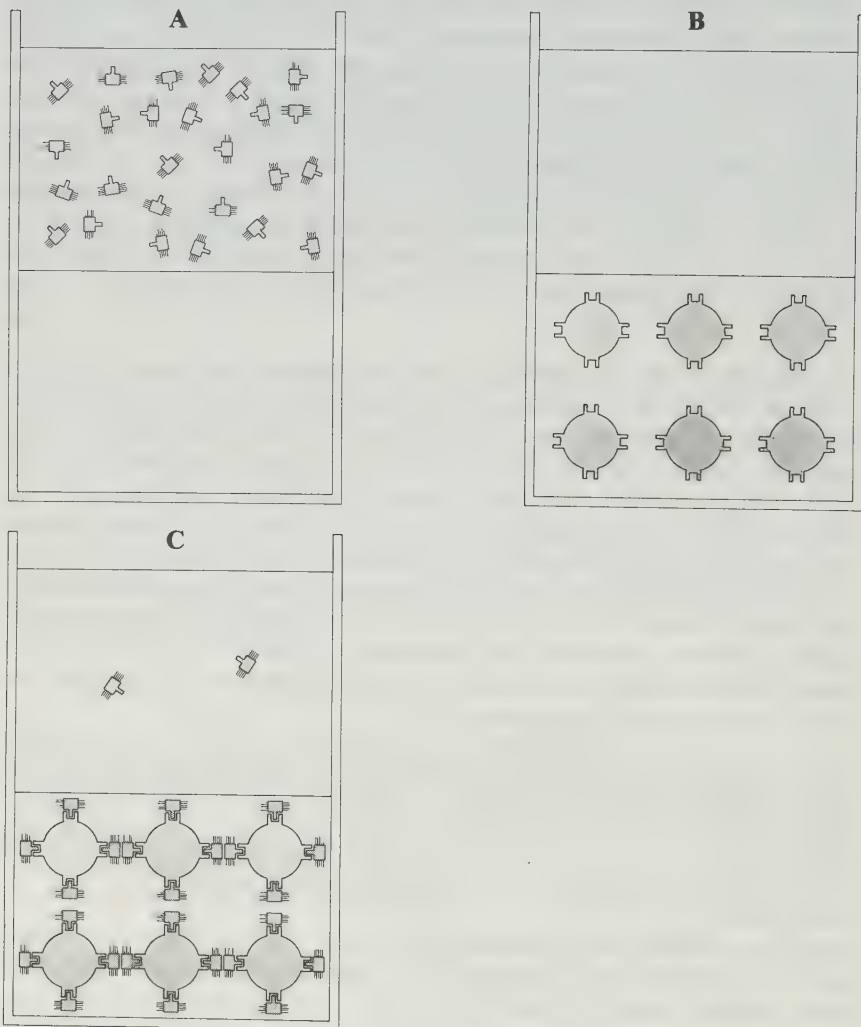


Figure 5. A schematic illustration of the principle of a direct binding assay using the PALA-principle.

A. M-PEG-modified, labelled antibodies partition mainly to the top phase. B. and C. Unmodified cells to be quantitated, partition mainly to the bottom phase, even in the presence of M-PEG-modified antibodies, resulting in lowered activity in the top phase.

QUANTITATIVE ANALYSIS OF *Staphylococcus aureus* BACTERIAL CELLS WITH PALA-TECHNOLOGY

The quantitative analysis of *Staphylococcus aureus* cells, using the pseudo-immunological interaction between protein A on the cell surface of *Staphylococcus aureus* (strain Cowan I), and human immunoglobulin G, was chosen as a model system in order to evaluate whether or not the PALA-principle could be utilized not only for analysis of low and high molecular weight substances, but also for cells (paper III).

When partitioning *Staphylococcus* cells alone in a phase system comprised of PEG and magnesium sulfate, the major fraction of cells were partitioned to the salt-rich bottom phase. The same partition pattern was obtained when human IgG was partitioned alone in a similar system. Thus, in order to be able to set up an assay, one of the reactants had to be modified to get the desired unsymmetrical partitioning. Suitable results may be obtained in two ways:

- * Monomethoxy poly (ethylene glycol) (M-PEG) modification of human IgG.
- * M-PEG modification of *Staphylococcus* cells.

When modifying the IgG-molecules it is possible to perform a direct binding assay, whereas modification of the cells requires the use of the competitive binding assay. In both cases studied the human IgG-molecules were labelled with the radioactive isotope ^{125}I .

Direct binding assay of *Staphylococcus aureus* cells

The direct binding assay was performed by the incubation of a sample containing an unknown number of *Staphylococcus aureus* cells together with a predetermined amount of M-PEG-modified, ^{125}I -labelled IgG-molecules. After incubation, a thoroughly mixed phase system, comprised of PEG and magnesium sulfate, was added to the mixture. The phases were allowed to settle, after which a sample was taken from the top phase for measurement of labelled antibodies. Figure 5.c schematically represents the analytical situation obtained after the addition of the phase system. A typical standard curve in a direct binding assay of *Staphylococcus aureus* cells is given in Figure 6. Cell numbers in the region 10^6 to 10^7 could be analyzed.

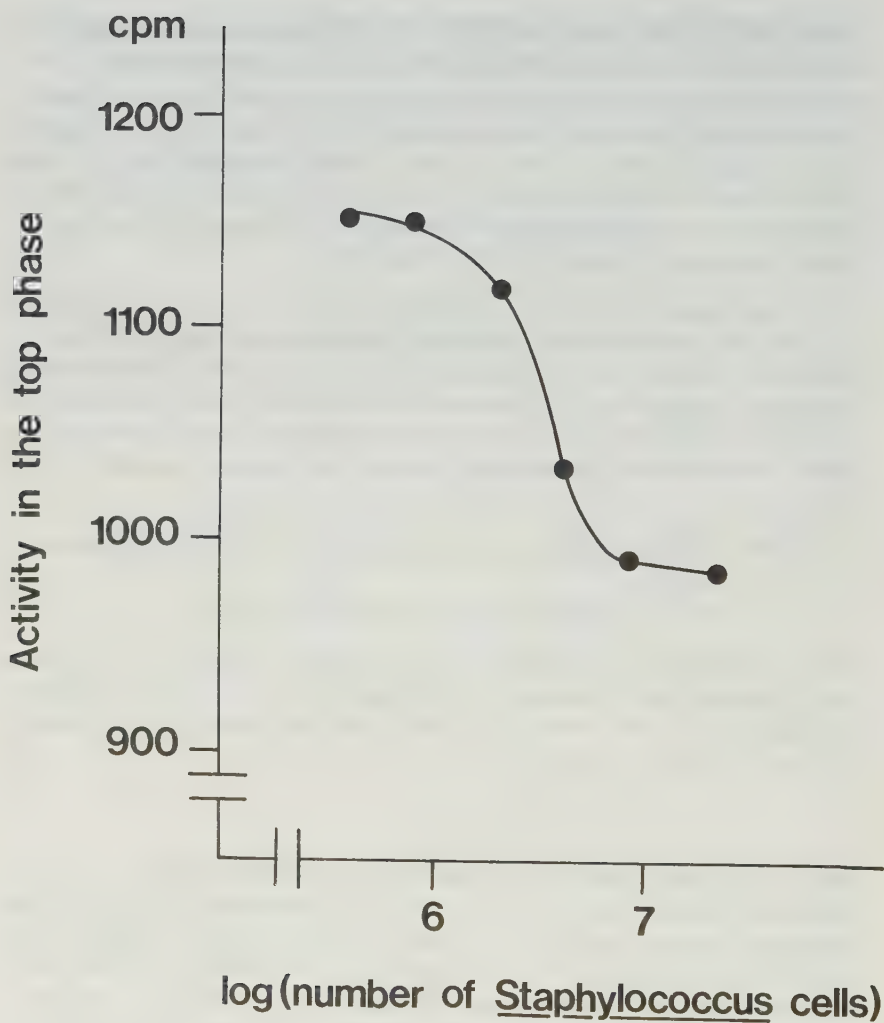


Figure 6. A calibration curve for *Staphylococcus aureus* using a direct PALA. The radioactivity in a sample taken from the top phase is given as a function of the cells in the incubation sample.

Competitive binding assay of *Staphylococcus aureus* cells

A competitive assay is performed by incubation of a known number of PEG-modified *Staphylococcus aureus* cells, mixed with an unknown number of unmodified cells, together with a predetermined amount of ^{125}I -labelled IgG. The basis of this assay is that the labelled antibodies should bind to the PEG-modified cells. If native cells are also present, competition for the labelled ligands should take place.

Figure 7 shows a schematic representation of a competitive binding assay. The PEG-modified cells partition mainly to the PEG-rich top phase, the labelled IgG-molecules to the bottom phase. The unmodified cells to be quantified also partition to the bottom phase. Incubation of PEG-modified cells together with labelled IgG, followed by partition in a phase system, results in an increased number of labelled IgG in the top phase. Addition of unmodified cells to the incubation mixture will, upon partition, lead to a reduction of the number of labels found in the top phase. Figure 8 shows a typical standard curve obtained in a competitive binding assay with *Staphylococcus aureus* cells. Cell numbers in the region 10^5 to 10^7 could be analyzed.

Quantitative analysis of *Streptococcus B₁* cells with PALA-technology.

Additional improvement of the sensitivity of an assay can be obtained if the signal from the label can be further amplified, for instance by employing a marker enzyme as label. In order to study this possibility, an effort to quantify *Streptococcus B₁* cells was performed using radiolabelled, as well as enzyme-labelled, anti-*Streptococcus* antibodies (paper IV).

The experimental principle of this assay is the same as that described in Figure 7, i.e. a competitive situation between native and modified cells for the binding of labelled antibodies occurs. In the study performed on *Streptococcus* cells, the phase system used was comprised of PEG and dextran. A calibration curve for the competitive assay is given in Figure 9. Cell numbers in the region 25 000 to 250 000 could be quantitated.

A unexpected effect was obtained when anti-*Streptococcus* antibodies were labelled with horse radish peroxidase according to the periodate method (Wilson and Nakane, 1978). The labelled antibodies partitioned alone to the PEG-rich phase. Since the native cells to be quantified partitioned to the bottom phase in a similar system, it suggested the possibility of performing a direct binding assay according to the principle presented in Figure 5. A pre-

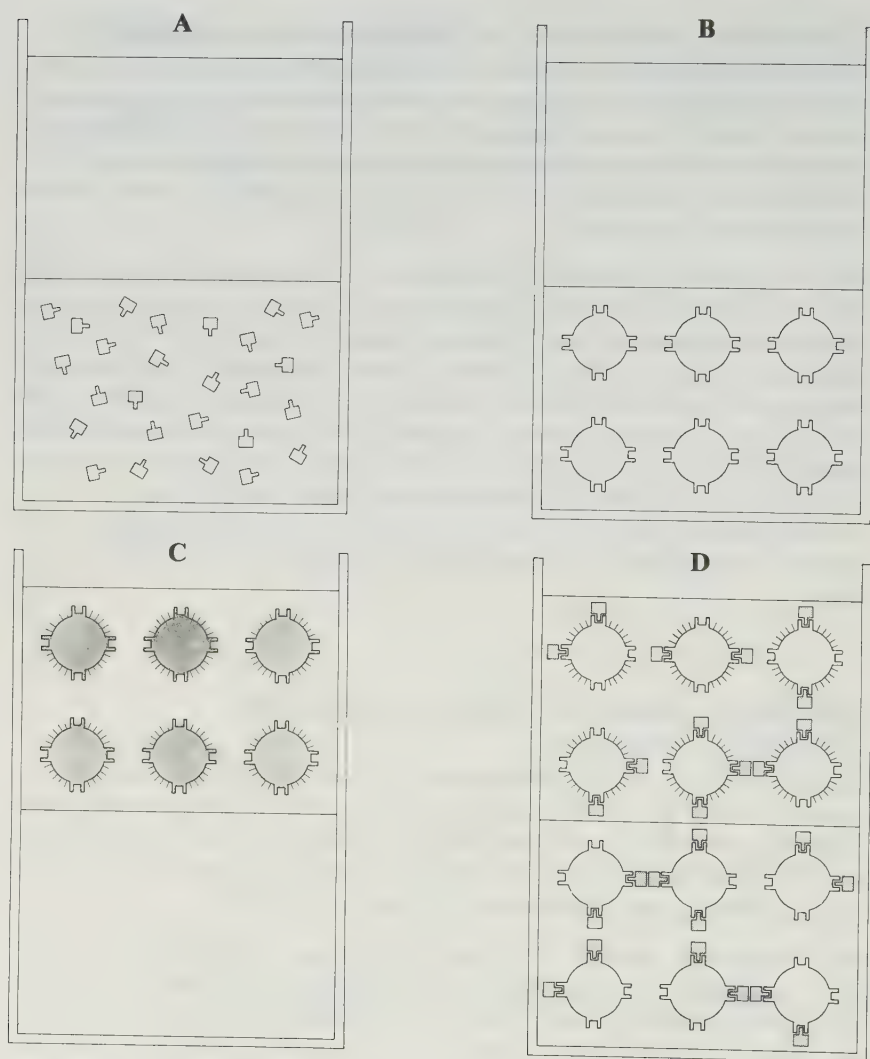


Figure 7. A schematic illustration of the principle of a competitive PALA.

A. Unmodified, labelled antibodies partition mainly to the bottom phase.

B. Unmodified cells to be quantitated, partition mainly to the bottom phase.

C. Modified cells partition mainly to the top phase.

D. Since competition between unmodified and M-PEG-modified cells for labelled antibodies takes place in the incubation mixture, a shift in the distribution of labelled antibodies occurs on addition of unmodified cells.

determined amount of enzyme-labelled antibody was incubated with a sample containing an unknown number of *Streptococcus* cells, followed by the addition of a well mixed phase system. When the phases had settled, a sample was taken from the top phase and analyzed for enzyme activity. Figure 10 shows a standard curve obtained in such an assay. Cell numbers in the region 2 500 cell to 250 000 could be quantitated.

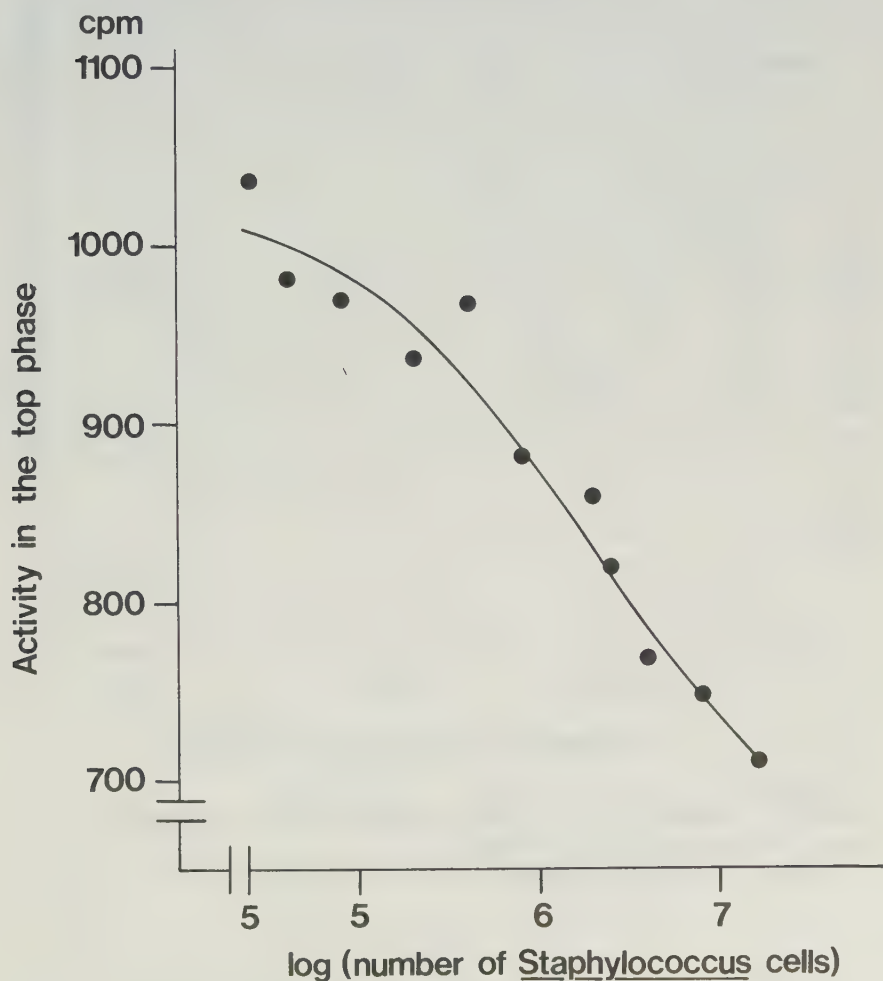


Figure 8. A standard curve for *Staphylococcus aureus* obtained in a competitive PALA: The activity in a sample taken from the top phase is given as a function of the number of cells present in the sample.

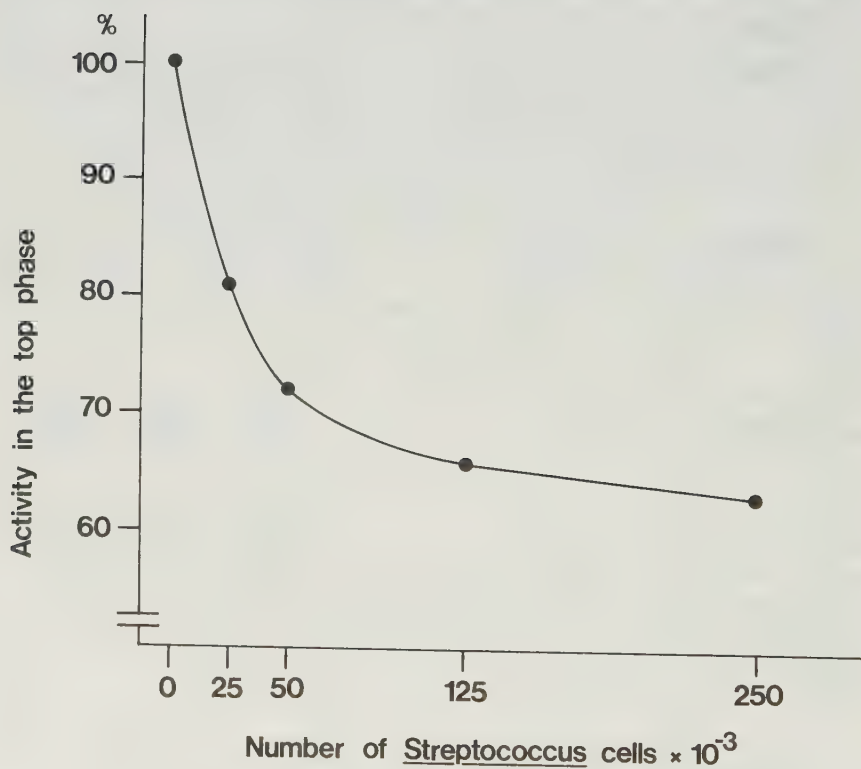


Figure 9. Standard curve for *Streptococcus* cells in a competitive PALA using radioactive labelled antibodies.

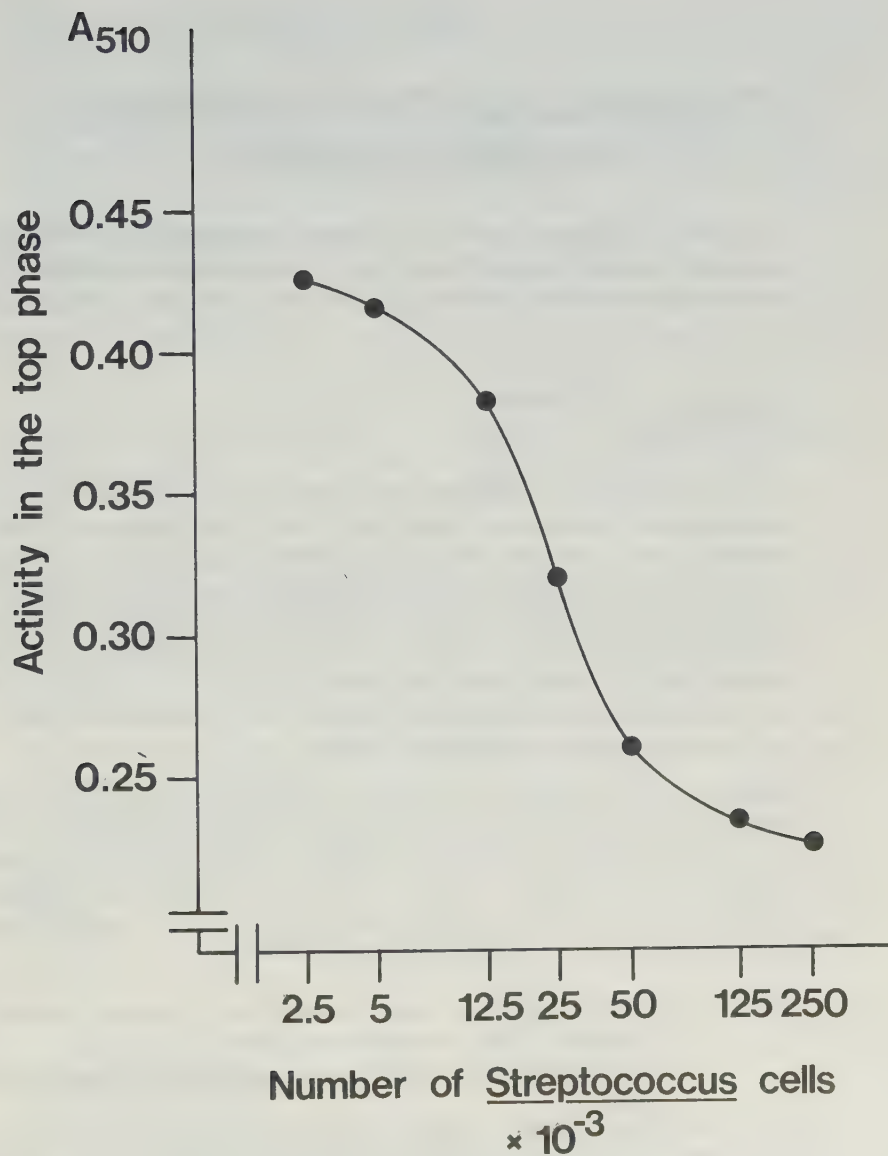


Figure 10. Standard curve for *Streptococcus* cells in a direct PALA using enzyme labelled antibodies.

ULTRAFILTRATION AFFINITY PURIFICATION

The affinity purification system described in paper V deals with the purification of the lectin Concanavalin A (Con A) from *Canavalia ensiformis*, using carbohydrate structures on the surface of heat killed yeast cells as ligands.

Lectins

The lectins are a group of proteins that more or less specifically interact with carbohydrates. Lectins have been known since 1888 (Stillmark, 1888), but gained no greater interest until it was discovered that the lectin in *Phaseolus vulgaris* was shown to have mitogenic activity (Nowell, 1960). Later it was shown that the lectin from wheat germ preferentially agglutinated malignant transformed cells (Aub *et al.*, 1963). The main characteristic of lectins is their ability to interact with carbohydrate residues on cell surfaces, leading to agglutination. The carbohydrate binding ability has made it possible to use lectins in various applications, Table 8.

Biological and biochemical research has found increased use for lectins, thereby prompting the development of new and powerful methods for their purification. The most commonly utilized purification technique is affinity chromatography, which utilizes appropriate carbohydrate residues as ligands, according to the specificity of the lectins. The chromatographic materials generally used for this purpose are:

- * Polysaccharide gel materials, such as Sephadex (Agrawal and Goldstein, 1967) or Sepharose (Gordon *et al.*, 1973).
- * Polysaccharides coupled to support material (Olson and Liener, 1967).
- * Glycoproteins coupled to support material (LeVine *et al.*, 1972).
- * Synthetically prepared glycosides, coupled to support material (Ravenstin *et al.*, 1974).

Table 8. Various applications of lectins.

Application	References
Determination of blood group antigens	Prokop and Uhlenbruch, 1969
Affinity chromatographic purification of glucoproteins	Goldstein, 1972 Lis and Sharon, 1973 Roth , 1978
Blood cell separation	Li and Osgood, 1949 Shortman, 1974 Hammarström <i>et al.</i> , 1977
Quantitative determination of of carbohydrates	Borrebaeck and Mattiasson, 1979 Mattiasson and Ling, 1980

Most of the affinity materials listed above are both time consuming and hard to synthesize. Furthermore, the obtained affinity adsorbents generally exhibit low binding capacities.

Lectins are normally identified by their ability to agglutinate red blood cells, but other cells can also be agglutinated, such as yeast cells, *Saccharomyces cerevisiae*. It has therefor been quite natural to immobilize such cells in order to employ them as affinity adsorbents for the purification of lectins. Reports have been published with respect to lectin purification using formaldehyde treated erythrocytes (Fauconnier, 1959), stromas entrapped in insolubilized albumin (Avrameas and Guilbert, 1971) or crosslinked by glutaraldehyde (Lawny *et al.*, 1978) and yeast cells entrapped in polyacrylamide (Mattiasson and Ramstorp, 1980) as well as in hollow fibers (Paper V).

Ultrafiltration

Ultrafiltration (UF) is a pressure activated membrane filtration process used for the separation and/or purification of dissolved or suspended particles. The membranes employed for UF have randomly distributed pores in a generally rigid and highly voided structure. The separation ability of UF-membrane is primarily based on particle size, such that molecular species larger than the largest pore are completely retained by the membrane, whereas species smaller than the smallest pore are completely permeable to the

membrane. Particles of intermediate size are partially retained in accordance to the pore size distribution. Thus, an UF-membrane functions analogously to a sieve, but on a molecular level (Cross and Strathmann, 1973; Michaels, 1968).

Pore size distributions are generally in the region 10 to 200 Å. Furthermore, UF-membranes can not distinguish between dimensionally similar substances such as, for example, water and sodium chloride. However, UF-membranes can readily distinguish between macro- and micromolecules, for instance a protein and a salt. Such membranes can be employed as semipermeable barriers separating two bulk solutions from one another, allowing transport of permeable molecules and preventing transport of larger molecules. This phenomenon has been utilized in chemical reactions involving one or more reactants, such as the stirred tank enzymatic reactor connected to a continuous recirculating ultrafiltration unit. In such a process the membrane quantitatively retains the enzymes/cells, while substrates for the reaction, as well as products, are readily permeable. This then allows continuous conversion of substrate into product and simultaneously allows quantitative recovery of the products without loss of enzymes/cells (Deeslie and Cheryan, 1981).

Another approach to utilizing membrane technology is the immobilized enzymatic wall hollow fiber membrane system, in which the catalyst, a concentrated enzyme solution or cell suspension, is confined in a hollow fiber unit. The immobilization is performed either with the catalyst free in solution or confined within the pores of the wall of an asymmetric hollow fiber membrane. Further improvements on this technology have been published. One example is to chemically couple catalysts to the surface of hollow fiber membranes (Michaels, 1980).

Ultrafiltration affinity purification of Concanavalin A

The experimental set-up for ultrafiltration affinity purification, as performed in paper V, is schematically illustrated in Figure 11. The system is comprised of a hollow fiber unit through which the cells are recirculated, a pressure meter, a level monitor and several pumps. The cells are recycled through the inner parts of the hollow fibers (A), and back to a beaker (B), by means of a peristaltic pump (C), which is controlled by a pressure meter (D) in order to avoid harmful pressures. The cells are washed with a buffer

suitable for the binding of the lectin, and after some time for equilibration to occur, a crude extract of *Canavalia ensiformis* is added to the cell suspension by means of a pump (E) controlled by a level monitor (F) which keeps the volume of the recirculation stream at a constant level. The outlet from the membrane, i.e. the solution penetrating the membrane, is passed through a photometer (G) for the continuous registration of the 280 nm absorbance in this stream.

After all of the extract has entered the recirculation stream, non-interacting materials are washed out with equilibration buffer. When all of the non-interacting material has left the system, i.e. absorbance at 280 nm is zero, material bound to the cells is eluted with a buffer containing glucose. The glucose competes with the structures on the cells for the lectin binding sites. Fractions are collected during the entire process and analyzed for the presence of lectin using an enzyme linked immunosorbent assay (ELISA). The biological activity of the lectin is also checked using an agglutination test.

The results obtained indicate that the non-interacting material contained some lectin which was proven to be biologically inactive, i.e. could not agglutinate red blood cells. The glucose eluted fractions, on the other hand, contained most of the lectin and this lectin was biologically active. SDS-PAGE showed that the fractions collected after glucose elution contained only one band.

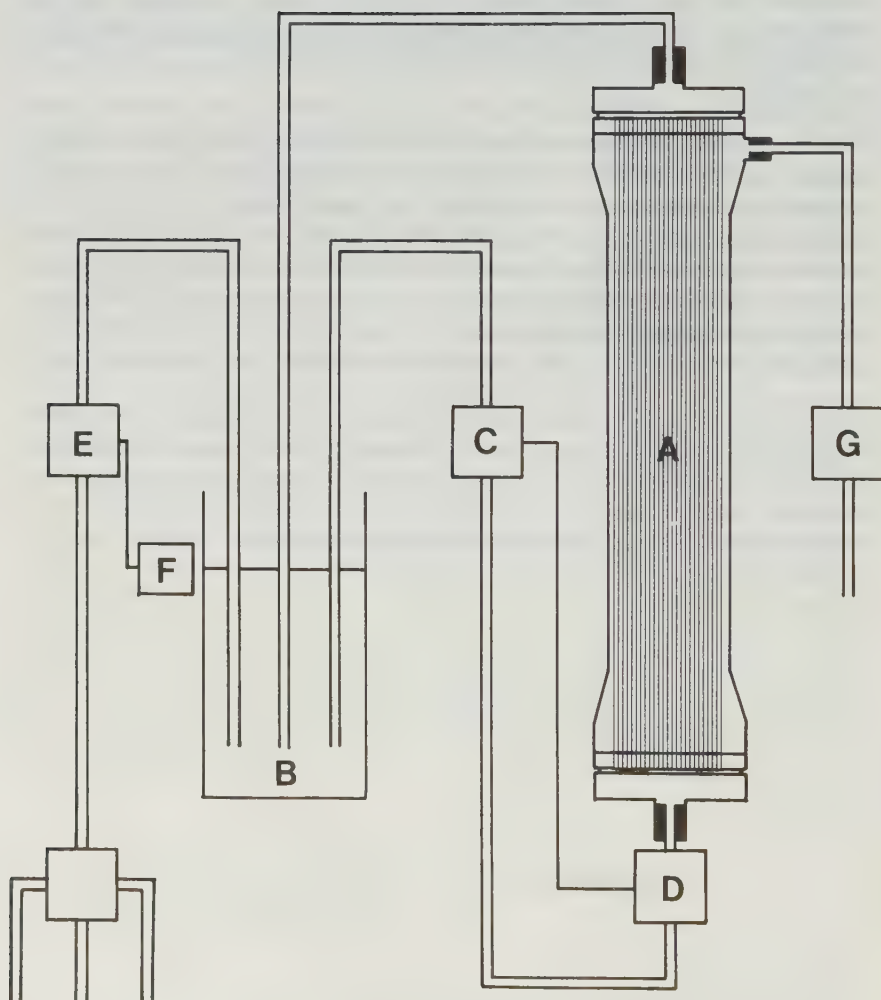


Figure 11. Schematic illustration of the experimental set-up for ultrafiltration affinity purification of Concanavalin A. Details are given in the text.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA).

Some examples of analytic techniques utilizing affinity interactions have been mentioned in the introduction. Immunological interactions between antibodies and antigens, as well as other interactions (for example between lectins and carbohydrate) can be utilized for the quantitation of one of the binding entities. Immunoassays based on the use of enzymes as labels have found increasing use in clinical laboratories.

Enzyme immunoassays can generally be divided into two types depending on whether or not the unbound, labelled molecules has to be washed away before identification can take place:

- * Heterogeneous enzyme immunoassays, including enzyme linked immunosorbent assay (ELISA).
- * Homogeneous enzyme immunosorbent assays, i.e. enzyme multiplied immunoassay (EMIT).

One major difference between these two techniques is that bound and free label has to be separated in heterogeneous assay, but not in homogeneous assays. The discussion below will be limited to ELISA-methods, which have been shown to be applicable for analysis of nearly all types of antigens.

Competitive enzyme immunosorbent assay

Antibodies with affinity for the antigen to be measured are bound to a solid support, either spherical beads or to the walls of plastic tubes, Figure 12. After removal of unbound antibodies, a mixture of an unknown amount of antigen and a predetermined amount of labelled antigen is added. After equilibrium and washing, the bound antigen containing label is visualized by incubation with an appropriate enzyme substrate solution. The amount of product formed via enzyme catalysis can be correlated to the amount of unlabelled antigen present in the incubation sample. It follows that the amount of antibody binding sites must be limited to make the assay work properly.

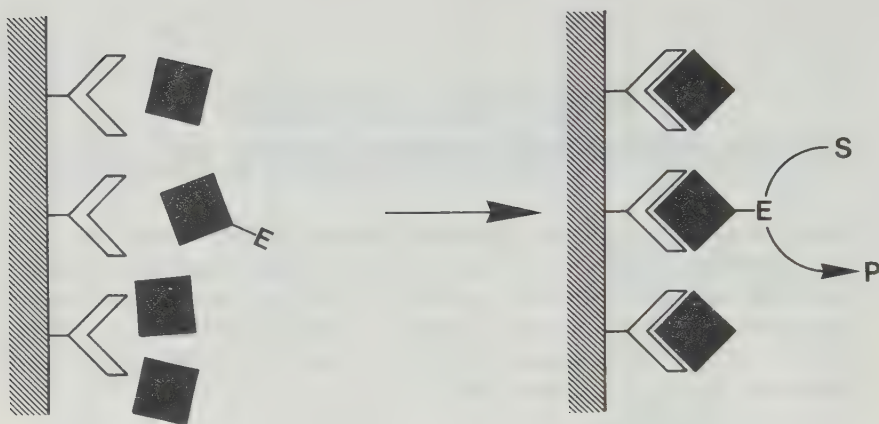


Figure 12. Schematic representation of a competitive enzyme immunoassay.

Non-competitive enzyme immunosorbent assays

The competitive immunosorbent assay described above can be performed independently of the number of antigenic determinants present on the antigen, as long as the antibody can interact with the antigen of interest. This is not the case with the non-competitive assay, which requires at least two antigenic determinants on the antigen.

As in the competitive immunosorbent assay, antibodies are coupled to a solid support material, Figure 13. After incubation, followed by washing, antibodies containing enzyme markers as labels are incubated. After another washing step, the amount of label is determined by incubation with an enzyme substrate solution. The amount of product formed can then be correlated to the amount of antigen present in the incubation mixture.

In the study of ultrafiltration affinity purification of Concanavalin A (paper V), a non-competitive ELISA was developed in which the sandwich antibody was replaced by the glycoprotein horse radish peroxidase. The analytical procedure for quantitation of Con A thus involved the incubation of samples containing Con A in wells coated with anti-Concanavalin A antibodies, followed by incubation with a peroxidase solution. The peroxidase bound to the Con A present in the wells was visualized by incubating with a solution of a chromogenic peroxidase substrate solution.

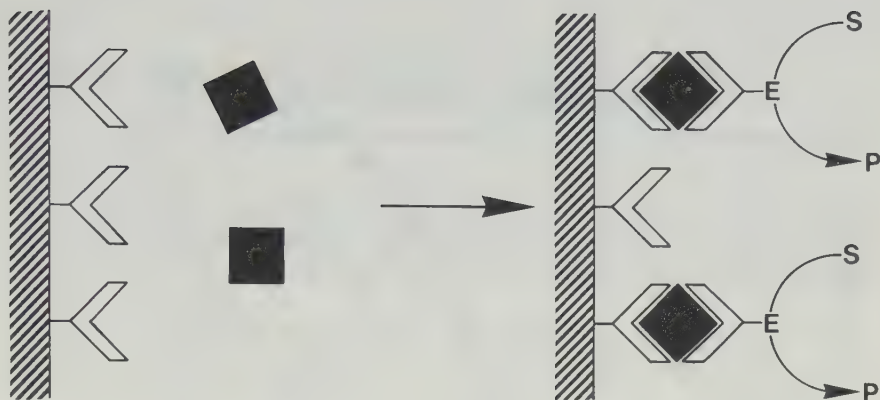


Figure 13. Schematic representation of a non-competitive enzyme immunoassay.

In another study (Ramstorp *et al.*, 1983) the main purpose was to determine coupling yield, ligand leakage and IgG-binding capacity of protein A coupled to tresyl chloride activated Sepharose. For this purpose, both competitive as well as non-competitive ELISA-methods were developed.

Figure 14 and 15 show the results obtained in the various assays developed for the quantitation of protein A and IgG, respectively. From these results it was concluded that the non-competitive alternative should be used when lower concentrations are to be analyzed. This is especially the case when determining the leakage of protein A from tresyl chloride activated Sepharose, where concentrations as low as 10 ng per ml should be assayed. However, an advantage of the competitive assay is that it only takes about half the time and is cheaper to perform if monoclonal antibodies are utilized. This is due to the fact that generally two monoclonal antibodies have to be available in order to perform a non-competitive ELISA.

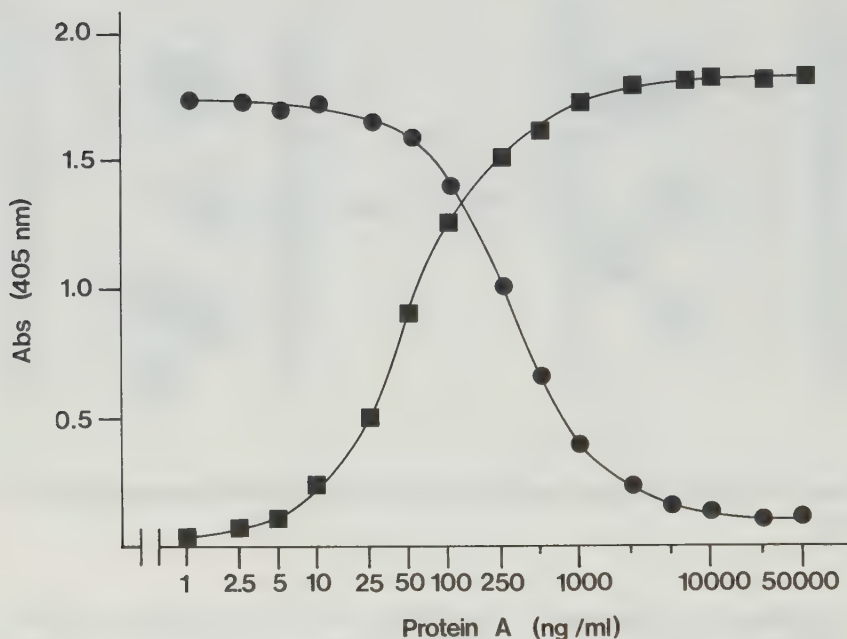


Figure 14. Results from the two ELISA-methods studied in connection with the analysis of protein A. • competitive ELISA; ■ non-competitive ELISA

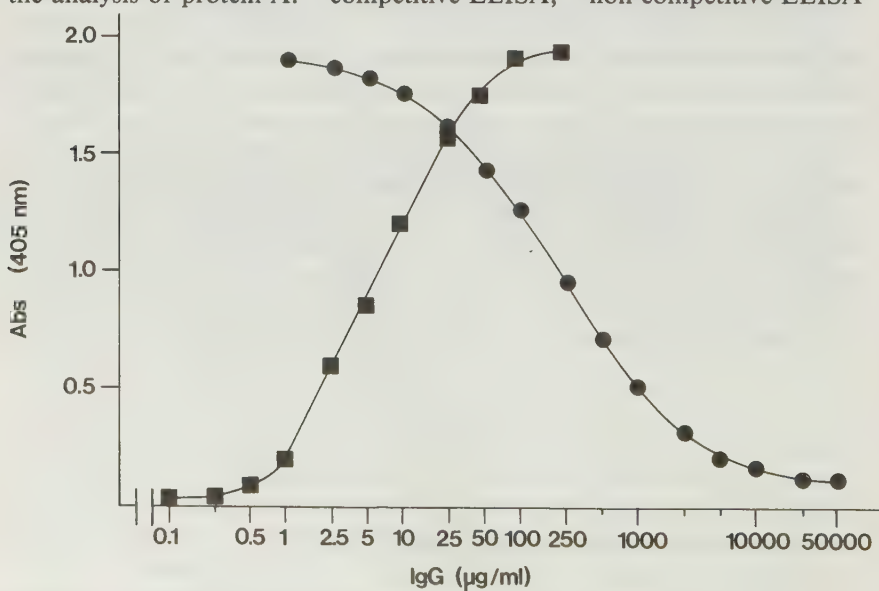


Figure 15. Results from the two ELISA-methods studied in connection with the analysis of IgG. • competitive ELISA; ■ non-competitive ELISA

CONCLUDING REMARKS

From the various experiments discussed, the following general conclusions may be drawn:

- * Cells immobilized in polyacrylamide can be used in affinity chromatographical applications for the study and purification of molecules interacting with surface structures on the immobilized cells.
- * Separation of free from bound labelled antibodies, by differences in partitioning in aqueous two phase systems, can be used for the quantitation of bacterial cells.
- * The combination of ultrafiltration technology with affinity interactions results in a powerful purification method, which may be performed in a continuous manner.

During the past two decades there has been an increasing utilization of affinity interactions for various applications. Due to the great variety and to the multiplicity of biospecific affinity interactions the possibility for even wider use in the future are numerous. The introduction of monoclonal antibodies being a promising approach in increasing the selectivity of biospecific analysis and purification systems.

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REFERENCES

A

- Abbott, B.J. in "Annual Reports on Fermentation Processes" (D. Perlman and G.T. Tsao, Eds.) vol. 1., Academic Press 1977, p. 205
- Abbott, B.J. in "Annual Reports on Fermentation Processes" (D. Perlman, Ed.) vol. 2., Academic Press 1978, p. 91
- Adler, F.L. and Liu, C.-T. (1971) *J. Immunology*, **106**, 1684
- Agrawal, B.B.L. and Goldstein, I.J. (1967) *Biochim. Biophys. Acta*, **147**, 262
- Albertsson, P.-Å. (1958) *Biochim. Biophys. Acta*, **27**, 378
- Albertsson, P.-Å. (1965) *Biochim. Biophys. Acta*, **103**, 1
- Albertsson, P.-Å., "Partition of Cell Particles and Macromolecules", 2nd Ed., Almqvist and Wiksell 1971, Sweden
- Albertsson, P.-Å., in "Cell Separation Methods" (H. Bloemendal, Ed.), Elsevier, Amsterdam 1977a, p. 79
- Albertsson, P.-Å. (1977b) *Endeavour*, **1**, 69
- Andersson, B. and Åkerlund, H.E. (1978) *Biochim. Biophys. Acta*, **503**, 462
- Andersson, B., Åkerlund, H.E. and Albertsson, P.-Å. (1976) *Biochim. Biophys. Acta*, **423**, 122
- Aub, J.C., Tieslau, C. and Lankester, A. (1963) *Proc. Nat. Acad. Sci. U.S.A.*, **50**, 613
- Avrameas, S. and Guilbert, B. (1971) *Biochimie*, **53**, 603
- Axén, R., Porath, J. and Ernback, S. (1967) *Nature*, **214**, 1302
- ### B
- Backman, L. and Johansson, G. (1976) *FEBS Lett.*, **65**, 39
- Beijerneck, M.N. (1896) *Zentralbl. Bacteriol.*, **2**, 627
- Birnbaum, S., Pendleton, R., Larsson, P.-O. and Mosbach, K. (1981) *Biotechnol. Lett.*, **3**, 343
- Birnbaum, S., Larsson, P.-O. and Mosbach, K. in "Solid Phase Biochemistry, Analytical and Synthetic Aspects" (W.H. Scouten, Ed.) Wiley 1983, in press
- Borrebaeck, C. and Mattiasson, B. in "Proteins of Biological Fluids" (H. Peeters, Ed.) vol. 27, Pergamon Press 1979, p. 607
- Brandt, J., Andersson, L.O. and Porath, J. (1975) *Biochim. Biophys. Acta*, **386**, 196
- Brattshall, D. in "Secretory Immunity and Infection" (J.R. McGhee, J. Mestecky and J.L. Babb, Eds.) Plenum Press 1978, p. 327
- Brodelius, P. and Mosbach, K. (1973) *FEBS Lett.*, **35**, 223
- Bøg-Hansen, T.C. (1973) *Anal. Biochem.*, **56**, 480
- ### C
- Chambell, D.H., Luescher, E. and Lerman, L.S. (1951) *Proc. Nat. Acad. Sci. U.S.A.*, **37**, 575
- Chang, T.M.S. (1964) *Science*, **146**, 524
- Chenais, F., Virella, G., Patric, C.C. and H.H. Fudenberg (1977) *J. Immunol. Methods*, **18**, 183
- Chibata, I. and Tosa, T. in "Annual Review of Biophysics and Bioengineering" (L.J. Mullins *et al.*, Eds.) Annual Reviews 1981, p. 197
- Chibata, I., Tosa, T. and Sato, T. (1974) *Appl. Microbiol.*, **27**, 878
- Coloe, P.J. in "Rapid Methods and Automation in Microbiology" (H.H. Johnston and S.W.B. Newson, Eds.) Research Studies Press, Oregon 1977, p. 27

- Coonrod, J.D. and Rytel, M.W. (1978) *J. Lab. Clin. Med.*, **81**, 770
- Cross, R.A. and Strathmann, H. in "Introduction to Separation Science" (B.L. Karger, L.R. Snyder and C. Horvath, Eds.) Wiley Interscience 1973
- D**
- Deeslie, W.D. and Cheryan, M. (1981) *J. Food Sci.*, **46**, 1035
- De Maeyer-Guignard, J., Tovey, M.G., Gresser, I. and De Maeyer, E. (1978) *Nature*, **271**, 622
- E**
- Easterday, R.L. and Easterday, I. in "Immobilized Biochemicals and Affinity Chromatography" (R.B. Dunlap, Ed.) Plenum Press 1974, p. 123
- Edelman, G.M., Rutishauser, U. and Milliet, C.F. (1971) *Proc. Nat. Acad. Sci. U.S.A.*, **68**, 2153
- Edwards, E.A. and Larsson, G.L. (1974) *Appl. Microbiol.*, **28**, 972
- Engvall, E. and Perlmann, P. (1971) *Immunochem.*, **8**, 871
- Erikson, I. (1974) *Biochim. Biophys. Acta*, **356**, 100
- Eriksson, E. and Albertsson, P.-Å. (1978) *Biochim. Biophys. Acta*, **507**, 425
- Eriksson, E., Albertsson, P.-Å. and Johansson, G. (1976) *Mol. Cell Biochem.*, **10**, 123
- Ersson, B. (1980) *Biotechnol. Bioeng.*, **22**, 79
- Ey, P.L., Prowse, S.J. and Jenkins, C.R. (1978) *Immunochem.*, **15**, 429
- F**
- Fauconnier, B. (1959) *Ann. Inst. Past.*, **96**, 110
- Finlay, T.H., Troll, V., Levy, M., Johnson, A.J. and Hodgins, L.T. (1978) *Anal. Biochem.*, **87**, 77
- Flanagan, S.D., Barondes, S.H. and Taylor, P. (1976) *J. Biol. Chem.*, **251**, 858
- Fornstedt, N. and Porath, J. (1975) *FEBS Lett.*, **57**, 187
- Forsgren, A. and Sjöquist, J. (1966) *J. Immunol.*, **97**, 822
- Forsgren, A. and Sjöquist, J. (1967) *J. Immunol.*, **99**, 19
- Fukami, H. and Itano, H.A. (1976) *Biochemistry*, **15**, 3529
- Fukui, S., Tanaka, A. and Gelfi, G. in "Enzyme Engineering" (G.B. Broun, G. Manecke and L.B. Wingard, Jr, Eds.) vol. 4., Plenum Press 1978, p. 299
- G**
- Gardestrom, P., Erikson, I. and Larsson, C. (1978) *Plant. Sci. Lett.*, **13**, 231
- Ghosh, S.K., Grossberg, A.L., Kim, U. and Pressmann, D. (1978) *Immunochem.*, **15**, 345
- Giard, D.J., Loeb, D.H., Thilly, W.G., Wang, D.I.C. and LeVine, D.W. (1979) *Biotechnol. Bioeng.*, **21**, 433
- Goettsch, E. and Kendall, F.E. (1935) *J. Biol. Chem.*, **109**, 221
- Goldstein, I.J. (1972) *Methods Carbohydr. Chem.*, **6**, 106
- Gordon, J.A., Blumberg, S., Lis, H. and Sharon, N. (1973) *FEBS Lett.*, **24**, 193
- Grov, A., Myklestad, B. and Oeding, P. (1964) *Acta Pathol. Microbiol. Scand.*, **61**, 588
- H**
- Hackel, U., Klein, J., Megnet, R. and Wagner, F. (1975) *Eur. J. Appl. Microbiol.*, **1**, 291
- Hahn-Hägerdal, B., Mattiasson, B. and Albertsson, P.-Å. (1981) *Biotechnol. Lett.*, **3**, 53
- Hammarström, S., Hellström, U., Dillner, M.L., Perlmann, P., Perlmann, H., Axelsson, B. and Robertsson, . in "Affinity Chromatography" (O. Hoffman-Oestenhof, M. Breitenbach, F. Kollner, D. Kraft and O. Scheiner, Eds.) Pergamon Press 1977, p. 273
- Hartman, A. and Heilbroinn, E. (1978) *Biochim. Biophys. Acta*, **513**, 382
- Hemmingsen, S.H. (1979) *Appl. Biochem. Bioeng.*, **2**, 157

- Hjelm, H. (1975) *Scand. J. Immunol.*, **4**, 633
- Hjelm, H., Hjelm, H. and Sjöquist, J. (1972) *FEBS Lett.*, **28**, 73
- Hubert, P., Dellacherie, E., Neel, J. and Banlieu, E.E. (1976) *FEBS Lett.*, **65**, 169
- I**
- Inman, J.K. in "Methods in Enzymology" (W.B. Jakoby and M. Wilchek, Eds.) vol. 34, Academic Press 1974, p. 30
- J**
- Jack, T.R. and Zajic, J.E. (1977) *Biotechnol. Bioeng.*, **19**, 631
- Jankowski, W.J., von Muenchhausen, W., Suckowski, E. and Carter, W.A. (1976) *Biochemistry*, **15**, 5182
- Janson, J.C. in "Affinity Chromatography and Related Techniques" (T.C.J. Gribnau, J. Visser and R.J.F. Nivard, Eds.) Elsevier 1982, p. 513
- Jensen, K. (1959) Thesis, Munksgaard, Copenhagen
- Johansson, G. (1970) *Biochim. Biophys. Acta*, **222**, 381
- Johansson, G. (1974) *Mol. Cell. Biochem.*, **4**, 169
- Johansson, G. (1976) *Biochim. Biophys. Acta*, **451**, 517
- Johansson, G. and Westrin, H. (1978) *Plant Sci. Lett.*, **13**, 201
- Johansson, G., Hartman, A. and Albertsson, P.-Å. (1973) *Eur. J. Biochem.*, **33**, 379
- Johnston, H.H. and Newson, S.W.B. (Eds.) in "Rapid Methods and Automation in Microbiology", Research Studies Press 1977
- K**
- Kaetsu, I., Kamakura, M. and Yoshida, M. (1979) *Biotechnol. Bioeng.*, **21**, 847
- Kahn, W., Friedman, G., Rodriguez, W., Controni, G. and Ross, S. in "Rapid Methods and Automation in Microbiology" (H.H. Johnston and S.W.B. Newson, Eds.), Research Studies Press 1977, p. 14
- Kan, J.K. and Shuler, M.L. (1978) *Biotechnol. Bioeng.*, **20**, 217
- Klein, J. and Eng, H. (1979) *Biotechnol. Lett.*, **1**, 171
- Klein, J. and Wagner, F. in "Enzyme Engineering" (H.H. Weetall and G.P. Royer, Eds.) vol. 5., Plenum Press 1980, p. 335
- Kobos, R.K., Rice, D.J. and Flournoy, D.S. (1979) *Anal. Chem.*, **51**, 1122
- Kronvall, G. (1973) *J. Med. Microbiol.*, **6**, 187
- Kronvall, G. and Williams, Jr., R.C. (1969) *J. Immunol.*, **103**, 828
- Kronvall, G., Simmons, A., Myhre, E.B. and Jonsson, S. (1979) *Infect. Immun.*, **25**, 1
- Kühn, I. (1980) *Biotechnol. Bioeng.*, **22**, 2393
- L**
- Lanner, M., Bergquist, R. and Carlsson, J. in "Affinity Chromatography" (O. Hoffman-Oestenhof, Ed.) Pergamon Press 1978, p. 237
- Larsson, C., Collin, C. and Albertsson, P.-Å. (1971) *Biochim. Biophys. Acta*, **245**, 425
- Larsson, P.-O. and Mosbach, K. in "Methods in Enzymology" (K. Mosbach, Ed.), vol. 44., Academic Press 1976, p. 183
- Larsson, P.-O., Ohlsson, S. and Mosbach, K. in "Applied Biochemistry and Bioengineering" (L.B. Wingard, E. Katchalski-Katir and L. Goldstein, Eds.) vol. 2., Academic Press 1979, p. 291
- Lartique, D.J. and Weetall, H.H. (1976) U.S. Patent 3.939.041
- Laurell, C.B. (1966) *Anal. Biochem.*, **15**, 45
- Lawny, F., Bot, M.-H., Lentwojt, E. and Segard, E. in "Affinity Chromatography" (O. Hoffman-Oestenhof, M. Breitenbach, F. Koller, D. Kraft and O. Scheiner, Eds.) Pergamon Press 1978, p. 299
- Lee, C.K. and Long, M.E. (1974) U.S. Patent 3.821.086

- Lesko, L., Donlon, M., Marinetti, G.V. and Hare, J.D. (1973) *Biochim. Biophys. Acta*, **311**, 173
- LeVine, D., Kaplan, M.J. and Greenoway, P.J. (1972) *Biochem. J.*, **129**, 847
- Li, J.G. and Osgood, E.E. (1949) *Blood*, **4**, 670
- Linko, P., Poutanen, K., Weckström, L. and Linko, Y.-Y. (1980) *Biochimie*, **62**, 387
- Lis, H. and Sharon, N. (1973) *Ann. Rev. Biochem.*, **42**, 541
- Löfkvist, T. and Sjöquist, J. (1962) *Acta Pathol. Microbiol. Scand.*, **56**, 295
- M**
- Magnusson, I. (1976) Thesis, Gothenburg
- Magnusson, K.E. and Johansson, G. (1977) *FEMS Microbial Lett.*, **2**, 225
- Mancini, G., Vaerman, J.P., Carabonera, A.O. and Hereman, S.J.F. in "Proteides of Biological Fluids" (H. Peeters, Ed.) vol. 11., Elsevier 1963, p. 370
- Mattiasson, B. and Eriksson, H. (1982) *Clin. Chem.*, **28**, 680
- Mattiasson, B. and Ling, T.G.I. (1980) *J. Immunol. Methods*, **38**, 217
- Mattiasson, B. and Ramstorp, M. in "Enzyme Engineering" (H.H. Weetall and C.P. Royer, Eds.) vol. 5., Plenum Press 1980, p. 401
- Mattiasson, B., Ramstorp, M., Nilsson, I. and Hahn-Hägerdal, B. (1981) *Biotechnol. Lett.*, **10**, 561
- Michaels, A.S. in "Progress in Separation and Purification" (E.S. Perry, Ed.) Interscience 1968.
- Michaels, A.S. (1980) *Desalination*, **35**, 329
- Mosbach, K. and Mosbach, R. (1966) *Acta Chem. Scand.*, **20**, 2807
- Mosbach, K., Guilford, H., Larsson, P.-O., Olsson, R. and Scott, M. (1971) *Biochem. J.*, **125**, 20
- Myhre, E.B. and Kronvall, G. (1980) *Infect. Immun.*, **27**, 6
- N**
- Neujahr, H.Y. and Kjellén, K.G. (1979) *Biotechnol. Bioeng.*, **21**, 671
- Nilsson, K. and Mosbach, K. (1980) *FEBS Lett.*, **118**, 145
- Nilsson, K. and Mosbach, K. (1981) *Biochem. Biophys. Res. Commun.*, **102**, 449
- Nilsson, H., Mosbach, R. and Mosbach, K. (1972) *Biochim. Biophys. Acta*, **268**, 253
- Nowell, P.C. (1960) *Cancer Res.*, **20**, 462
- O**
- Olson, M.O.J. and Liener, I.E. (1967) *Biochemistry*, **6**, 105
- Omata, T., Tanaka, A., Yamane, T. and Fukui, S. (1979) *Eur. J. Appl. Microbiol. Biotechnol.*, **6**, 207
- P**
- Pestka, S., Weiss, D. and Vince, R. (1976) *Anal. Biochem.*, **71**, 137
- Phillips, K.D., Tearle, P.Y. and Wills, A.T. in "Rapid Methods and Automation in Microbiology" (H.H. Johnston and S.W.B. Newson, Eds.) Research Studies Press 1977, p. 27
- Pickering, L.K. (1976) *J. Am. Med. Assoc.*, **236**, 1882
- Pinaev, G., Hoorn, B. and Albertsson, P.-Å. (1976) *Exp. Cell. Res.*, **98**, 127
- Porath, J. and Fornstedt, N. (1970) *J. Chrom.*, **51**, 479
- Prokop, O. and Uhlenbruch, G. in "Human Blood and Serum Groups" McLaren 1969, p. 72
- R**
- Ramstorp, M., Nilsson, K., Mosbach, R. and Mosbach, K. (1983) in preparation
- Ravenstin, M.E., Obrenovitch, A. and Monsigny, M. (1974) *FEBS Lett.*, **40**, 62
- Robinson, P.J., Wheatly, M.A., Janson, J.C., Dunnill, P. and Lilly, M.D. (1974) *Biotechnol. Bioeng.*, **16**, 1103
- Roth, J. in "The lectins - Exp. Pathol. Suppl. 3." Fisher Verlag 1978, p. 125
- Rytel, M.W. in "Rapid Diagnosis in Infectious Disease" (M.W. Rytel, Ed.) CRC-Press 1979a, p. 1
- Rytel, M.W. (Ed.) in "Rapid Diagnosis in Infectious Disease" CRC-Press 1979b

S

Sanderson, C.J. and Wilson, D.V. (1971) *Immunology*, 20, 1061

Sato, T., Nishida, Y., Tosa, T. and Chibata, I. (1979) *Biochim. Biophys. Acta*, 570, 179

Scheidegger, J.J. (1955) *Internat. Arch. Allergy Appl. Immunol.*, 7, 103

Shanbhag, V.P. and Axelsson, C.-G. (1975) *Eur. J. Biochem.*, 60, 17

Shanbhag, V.P. and Johansson, G. (1974) *Biochim. Biophys. Acta*, 61, 1141

Shapiro, D.J. and Schimke, R.T. (1975) *J. Biol. Chem.*, 250, 1759

Shortman, K. (1974) *Contemp. Top. Mol. Immunol.*, 3, 161

Sjöquist, J., Forsgren, A., Gustavsson, G.T. and Stålenheim, G. (1967) *Cold Spring Harbour Symp. Quant. Biol.*, 32, 577

Soderman, D.D., Germershausen, J. and Katzen, H.M. (1973) *Proc. Nat. Acad. Sci. U.S.A.*, 70, 792

Starkenstein, E. (1910) *Biochem. Z.*, 24, 210

Stendahl, O., Magnusson, K.-E., Tagesson, C., Cunningham, R. and Edebo, L. (1973) *Infect. Immun.*, 7, 573

Stillmark, H. (1888) Thesis, Dorpat

Sundberg, L. and Porath, J. (1974) *J. Chrom.*, 90, 87

T

Takata, I., Tosa, T. and Chibata, I. (1977) *J. Solid Phase Biochem.*, 2, 225

Tanaka, A., Jin, I.-N., Kawamoto, S. and Fukui, S. (1979) *Eur. J. Appl. Microbiol. Biotechnol.*, 7, 351

Toda, K. and Shoda, M. (1975) *Biotechnol. Bioeng.*, 17, 481

Tosa, T., Sato, T., Mori, T., Yamamoto, K., Takata, F., Nishida, Y. and Chibata, I. (1979) *Biotechnol. Bioeng.*, 21, 1697

Tsumura, N. and Kasumi, T. (1976) Abstract of paper, 5th International Fermentation Symposium, Berlin

V

Walter, H. in "Methods of Cell Separation" (N. Catsimpooolas, Ed.) vol. 1., Plenum Press 1977, p. 307

Walter, H. and Selby, F.W. (1966) *Biochim. Biophys. Acta*, 112, 146

Walter, H., Eriksson, G., Taube, Ö. and Albertsson, P.-Å. (1970) *Exp. Cell. Res.*, 64, 486

van der Loo, W. and Hamers, R. in "Proteins of Biological Fluids" (H. Peeters, Ed.) vol. 23., Pergamon Press 1976, p. 603

Weston, P.D. and Avrameas, S. (1971) *Biochem. Biophys. Res. Commun.*, 45, 1574

Westrin, H., Albertsson, P.-Å. and Johansson, G. (1976) *Biochim. Biophys. Acta*, 436, 696

Wichman, R., Wandry, C., Buckmann, A. and Kula, M.-R. (1981) *Biotechnol. Bioeng.*, 23, 2789

Vieth, W.R. and Venkatasubramanian, K. in "Enzyme Engineering" (G. Broun, G. Manecke and L.B. Wingard, Eds.) vol. 4., Plenum Press 1978, p. 307

Wigzell, H. and Andersson, B. (1969) *J. Exp. Med.*, 129, 23

Wilson, M.B. and Nakane, P.K. in "Immunofluorescence and Related Staining Techniques" (W. Knapp, K. Holubar and G. Wick, Eds.) Elsevier 1978, p. 215

Y

Yalow, R. and Berson, S. (1959) *Nature*, 184, 1648

Yoga, Y. and Wimmer, E. (1975) *J. Mol. Biol.*, 92, 467

I

Affinity Chromatography Using Immobilized Bacterial Cells with Receptors for Human Serum Proteins

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Affinity chromatography materials containing immobilized bacterial cells were prepared. Immobilization techniques of different types were developed and the products obtained were compared with respect to such properties as binding capacity, flow properties, and mechanical stability. Entrapment of bacteria in macroporous supports proved better than the use of surface-bound cells, both in terms of number of bacterial cells immobilized and flow characteristics of the product. Bacterial cells entrapped in a matrix of acrylamide crosslinked with *N,N'*-methylenebisacrylamide proved to be the best column material. To achieve the best properties of the product, polymerization was performed in two steps. In the first step small beads were formed and in the second step, they aggregated. This procedure for making bacterial cell columns allowed the use of *Staphylococcus aureus* strain Cowan I and three strains of β -hemolytic streptococci for affinity chromatographic purification of human serum proteins. Specific receptors present on the cells used cause interaction with serum proteins. Thus IgG binds to protein A and to similar Fc receptors on streptococci and albumin can bind to group C and G streptococci. The results reported indicate that bacterial cell columns with high-adsorption capacities and good flow characteristics can be made.

INTRODUCTION

Affinity chromatography has been developed during the last 10-15 years and is now a method used routinely for purifying proteins (1). Initially individual inhibitors were immobilized and used for purifying single substances. Recently more general methods have been developed. Thus, it is now possible to purify groups of enzymes by utilizing their inherent affinity for a specific ligand, usually a coenzyme (2) or a fragment thereof, or a specific dye (3). Separation of the proteins bound to such general ligands has been made possible by specific elution (4, 5) of the adsorbed proteins.

A parallel development has taken place in immunoaffinity chromatography although it has proven more difficult to find general ligands for use in this technique (6). An important advance in this area is the use of protein A as a general anchor with which antibodies may interact (7).

The development of affinity chromatography has been based on the use of pure, well-defined, native, or premodified ligands coupled to defined supports or coupled to a premodified support. The general approach has been to use chemically well-defined supports to eliminate nonspecific interactions (1).

Recently, purified chemically well-defined ligands have been replaced by more "crude" systems, e.g., cell walls carrying biochemical structures of high complexity (8–10). Results published to date have been obtained from experiments using rather poorly defined systems containing the immobilized biological structure, e.g., by adsorption. Loss of ligand during use is a major drawback of these methods.

This paper concerns the development of new affinity adsorption materials using immobilized whole microbial cells containing specific receptors for serum proteins, as exemplified in studies of the binding properties of *Staphylococcus aureus* and some streptococcal strains for serum proteins.

MATERIALS AND METHODS

Chemicals used. Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and ammonium persulfate, all obtained from Merck, Darmstadt, West Germany, were of analytical grade. Sepharose CL 4B was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden; Arlacel 83 from Atlas Chemie, Essen, West Germany; dimethylsuberimide dihydrochloride from Pierce Eurochemie B. V., Rotterdam, The Netherlands. All other chemicals used were of analytical grade.

Bacterial strains. *Staphylococcus aureus*, strain Cowan I (NCTC No. 8530) was used in the form of formaldehyde and heat-stabilized cells as described previously (11). Group A streptococcus strain AR 1 (our laboratory reference number) was obtained from the WHO Collaborating Centre for Reference and Research on Streptococci, Dr. J. Rotta, Prague, Czechoslovakia (strain No. 40/58, M-type I), and strain AW 43 (our laboratory reference number) was from Dr. L. W. Wannamaker, Minneapolis, Minnesota (strain No. GT 71-727). Group G streptococcus strain G-148 was obtained from a routine bacteriological specimen at the Clinical Microbiology laboratory, Lund, Sweden (12).

Preparation of acrylamide gel beads containing entrapped bacterial cells. The polymerization process for producing polyacrylamide gel beads containing entrapped bacterial cells is carried out in a liquid two-phase system (13). The hydrophilic phase of the system, the monomeric solution containing the catalytic system mixed together with the cell suspension, and the hydrophobic phase, toluene/chloroform, are stirred together allowing the hydrophilic phase to form small droplets in the hydrophobic phase. The monomers in these droplets polymerize within approximately 30 min forming a product of solid beads containing cells entrapped in the polyacrylamide network. To eliminate oxygen from the polymerization mixture nitrogen is continuously bubbled through the reaction mixture. The hydrophobic phase (100 ml) consists of a toluene-chloroform mixture (2.64/1.00, v/v) of the same density as the hydrophilic phase. The hydrophobic phase, which also contains 0.7 ml of a surfactant, Arlacel 83, is stirred slowly under a constant flow of nitrogen gas for 30 min before addition of the hydrophilic phase. The hydrophilic phase consists of two different components. The cell suspension is prepared by diluting 3.4 ml of a 10% (w/v) cell suspension in

PBS¹ to a total volume of 10.2 ml then cooling in an ice bath. The monomer solution is prepared by dissolving 1.17 g acrylamide and 456 mg BIS in PBS and adjusting to a total volume of 4.1 ml. The monomer solution is poured into the cell suspension, stirred, and exposed to nitrogen gas for 3 min. The catalytic system consisting of 50 μ l TEMED and 1.0 ml of an ammonium persulfate solution (75 mg/ml PBS) is added to the hydrophilic phase and the mixture is poured rapidly into an Erlenmeyer flask, in which the nitrogen-bubbled hydrophobic phase is stirred at high speed. After about 15 min, when the first small beads become visible, the stirring speed is decreased resulting in aggregation of the beads and the consequent formation of larger particles. The polymerization is completed within 30 min and the solid beads are separated from the liquid medium by decantation. The beads are poured into a 1000-ml beaker, where they are suspended in several large portions of lukewarm water containing a detergent. Finally, the beads are washed in PBS and stored in this buffer containing 0.02% sodium azide.

Preparation of immobilized bacterial cells by covalent linkage to Sepharose. The immobilization takes place in two steps; first, immunoglobulin G is coupled to Sepharose by the conventional CNBr method (14). The bacterial cells, which contain specific receptors for the Fc portion of the immunoglobulin, are then incubated with the gel containing immobilized IgG and finally crosslinked.

Swollen Sepharose CL 4B freed of access moisture (5.0 g) is washed on a glass filter with 150 ml 0.1 M NaCl and 150 ml distilled water, suspended in 5.4 ml 2.0 M Na₂CO₃ and poured into a beaker placed on a magnetic stirrer. The gel is activated for 10 min with 70 mg CNBr per gram of swollen gel, keeping the pH at 10.8 by dropwise addition of 2.0 M NaOH. The activated gel is transferred rapidly to a cooled glass filter and washed with 100 ml ice-cold 0.1 M NaHCO₃. Finally, the gel is suspended in 10 ml of 0.1 M NaHCO₃ solution.

Immunoglobulin G, 5.2 mg is dissolved in 2.0 ml water and incubated with the activated washed gel in a test tube. The incubation is allowed to proceed for 1 h at room temperature, with periodic agitation of the test tube. The gel is then washed with 200 ml of 0.1 M NaHCO₃, then with 200 ml of 0.5 M NaCl and finally with 200 ml of water. The IgG-containing gel is stored in PBS with 0.02% sodium azide.

Bacterial cell suspension (0.5 ml, 10%, w/v) is mixed with the IgG-Sepharose. After incubation for 1 h at room temperature, the biospecifically bound cell preparation is thoroughly washed with 200 ml PBS then the cells are covalently bound to the gel beads by the use of 0.1 M glutaraldehyde or dimethyl suberimidate, crosslinking with the latter reagent being performed by a method described in greater detail elsewhere (15). The gel containing bacterial cells is washed with PBS to remove sodium azide, suspended for 10 min in 20 ml borate buffer, centrifuged and treated for 30 min with a solution of 0.1 g dimethylsuberimidate in 20 ml borate buffer. The gel is washed with 250 ml PBS and kept overnight in 40 ml of a solution containing 0.15 M NaCl and 0.1 M acetic acid, pH 2.9, and then stored in PBS containing 0.02% sodium azide.

Preparation of alginate gel containing entrapped bacterial cells. The method described is based on the property of alginate to form a three-dimensional network when treated with a solution of Ca²⁺ ions. If a biological material is mixed with

¹ Abbreviations used: BIS, *N,N'*-methylenebisacrylamide; TEMED, *N,N,N',N'*-tetramethylethylenediamine; PBS, 0.1 M Na₂HPO₄/NaH₂PO₄ buffer, pH 7.4, containing 0.15 M NaCl; borate buffer, 0.1 M H₃BO₃/NaBO₂, pH 8.0; IgG, immunoglobulin G.

alginate, the material will be entrapped in the alginate network. After being washed with tap water the gel is stabilized by using a crosslinking agent.

The alginate solution is prepared by a method described elsewhere (16). Two kinds of alginate gels are prepared, the first containing only bacterial cells and the other containing collagen also. Bacterial cell suspension (0.25 ml, 10%, w/v) is added to a 25-ml beaker containing 10.0 g of 2.5% alginate solution. In the second of the two preparations, a well-homogenized collagen solution is also added to the alginate-cell suspension. The mixture is then thoroughly blended after which it is ready for gel formation.

Alginate beads are formed by forcing the mixture slowly through a thin syringe needle (diameter, 1.2 mm) and allowing the droplets formed to fall into a slowly stirred 0.1 M CaCl_2 solution. The beads solidify within approximately 10 min and are finally stabilized, after washing with tap water, by using 0.1 M glutaraldehyde solution.

Preparation of agarose gel beads containing entrapped bacterial cells. The immobilization of bacterial cells in agarose is based on the ability of an agarose suspension to form a three-dimensional network, in which the cells are entrapped, when the gel is cooled to below its solidifying temperature. To produce a gel of bead shape, the process is carried out in a liquid two-phase system, in which the hydrophobic phase, a mixture of toluene and chloroform, serves as a dispersion medium (17).

Bacterial cell suspension (1.0 ml, 10%, w/v) is added to 12.6 g of a 2% hot (55°C) agarose solution. The mixture is stirred vigorously for 30 s. This short stirring time is used to avoid any possible heat denaturation of the cell surface proteins on the bacterial cells. This mixture constitutes the hydrophilic phase of the system.

The hydrophobic phase consists of a toluene-chloroform solution with a density of 1.22 g cm^3 , the same density as that of the hydrophilic phase. To an Erlenmeyer flask containing 0.5 ml of a surfactant, Arlacel 83, 100 ml of the toluene-chloroform mixture is added. The hydrophilic phase is poured rapidly into the Erlenmeyer flask, in which the hydrophobic phase is stirred at high speed. The agarose mixture is dispersed into small droplets, and since the content of the flask has a temperature lower than that of the liquefied agarose, the small droplets coagulate and form solid gel beads. After stirring has been continued for 5 min, the beads are allowed to sediment. The gel beads are separated from the liquid medium by decantation and are then washed thoroughly with several large portions of lukewarm water containing a small amount of detergent. Finally, the gel is suspended in PBS containing 0.02% sodium azide.

Affinity chromatography. The equipment used for affinity chromatographic studies comprised a column (80 × 16 mm) with flow adaptors; a photometer, 2138 Uvicord S; a peristaltic pump, 12000 Variopex; and a fraction collector, 2112 Redirac. All equipment used was purchased from LKB, Bromma, Sweden.

The gel was packed in the column and was equilibrated with a continuous flow of PBS (0.45 ml/min). The sample was applied in the same buffer and when no absorbance was registered in the eluate, a pH gradient was applied. The vessel at the outlet of the gradient mixer contained 30 ml 0.1 M phosphate buffer, pH 7.0, and the other vessel contained, at the start, 30 ml of 0.2 M citrate buffer, pH 2.0. Fractions of 50 drops (2.9 ml) were collected. The gel was reused after equilibration with PBS.

Analysis of column eluates. Qualitative and quantitative analysis of the serum protein binding properties of columns were performed with immobilized streptococcal strains G-148, AR 1, AW 43, and *S. aureus* strain Cowan 1 as the absorbing material in crosslinked polyacrylamide. A mixture of 0.2 mg human serum albumin and 0.2 mg immunoglobulin G (lyophilized preparation, Kabi, Stockholm, Sweden) in 2 ml PBS was applied to the columns. The absorbance of the eluate at 280 nm was recorded continuously. Excess amounts of serum were chromatographed in a similar fashion. To identify individual proteins eluted by the pH gradient, the fractions obtained were dialyzed and then analyzed by the rocket immunoelectrophoretic technique (18) in gels of 0.8% agarose (Miles Laboratories, Ltd., England). Antibodies with defined specificities and antibody titers were used in the agarose gels, 0.5% rabbit anti-albumin and 0.5% rabbit anti-IgG, respectively (Dako A/S, Copenhagen, Denmark), with 0.075 M barbital buffer, pH 8.6, containing 0.002 M calcium lactate as the electrophoresis buffer. A potential of 50 V was applied for 18 h over the gel plates. After drying the plates were stained with Coomassie brilliant blue R-250 (Mann Research Laboratory, New York, N. Y.). Protein in fractions eluted from the column was determined according to Folin.

RESULTS AND DISCUSSION

Preparation of Immobilized Bacterial Cell Columns by Covalent Coupling

In preliminary experiments covalent coupling of *S. aureus* to CNBr-activated Sepharose was tried for preparing bacterial cell columns as well as for binding of the staphylococcal cells to IgG-Sepharose via protein A followed by subsequent crosslinking with dimethylsuberimide or glutaraldehyde. The results of these experiments involving anchoring of whole cells to the support matrix via covalent bonds showed the approach to be possible. However, very low yields of bound bacterial cells were obtained with the methods used and the cells tended to leak from the support.

Preparation of Immobilized Bacterial Cell Columns Using Entrapment Methods

Entrapment of cells, as an alternative to covalent binding, was tested in an effort to obtain gels with higher capacity, improved yield, and reduced leakage of cells. The support materials used for immobilization were agarose, polyacrylamide, alginate, and alginate-collagen (Table 1).

Agarose was liquefied and mixed with the bacterial cells before dispersion in an organic phase (17). The yield in this process and the mechanical and chromatographic properties of the products obtained proved good. By varying the stirring speed and/or the amount of detergent added, a particle size giving good flow properties was obtained. However, since the gel is not stabilized by crosslinking, the elution conditions used in the applications studied here (0.2 M glycine-HCl, pH 2.2) disturbed the gel structure. Furthermore, generally speaking, the necessity to heat the organism before immobilization is not desirable since even at 55°C some surface structures may be destroyed.

To eliminate the drawback associated with the last approach a very gentle entrapment technique was tried. The cells were mixed with alginate, which in a

TABLE I
Immobilization Procedures Tested

<i>Carrier</i>	<i>Immobilization method</i>	<i>Results</i>
Covalent coupling		
Sepharose CL 4B	CNBr activation	Low yield
IgG-Sepharose CL 4B	Adsorption and subsequent crosslinking with	
	a. Glutaraldehyde	Very low yield
	b. Dimethyl suberimidate	Low yield
Entrapment		
Agarose (2-4%)	Bead formation using an organic phase	High yields; product had low stability
Alginate (2.5%)	Beads formed by adding drops to a water solution of Ca^{2+} ions	High yields; low porosity; necessary to maintain a high level of Ca^{2+} throughout the whole procedure
Alginate-collagen	Copolymerization in a water solution of Ca^{2+} ions; then dissolution of the alginate	Low mechanical stability (may be improved); low yield
Crosslinked polyacrylamide	Bead polymerization	High yield; high capacity; good mechanical properties

subsequent step was crosslinked by exposure to 0.1 M Ca^{2+} (16). The support obtained in this manner retains its shape as long as the carboxyl groups of the support are crosslinked by the divalent metal ions. However, owing to the viscosity of the mixture, droplets smaller than 1 mm in diameter were not obtained. Good properties for chromatographic purposes requires a homogeneous distribution of rather small particles. At the present stage this characteristic is not attainable. Furthermore, the need for divalent metal ions to maintain the structure might not be acceptable in some applications.

To avoid these problems, yet another method, involving entrapment in mixtures of collagen and alginate, was tried. The beads were produced by exposing the mixture to 0.1 M Ca^{2+} . After the beads had been harvested, the collagen was crosslinked by glutaraldehyde prior to removal of alginate from the beads by washing with phosphate buffer containing EDTA to eliminate the Ca^{2+} required for maintenance of the alginate gel structure. Porous spherical collagen beads containing immobilized bacteria were obtained. However, besides the size of the beads being undesirable, the use of these preparations in practice was also hampered by the low mechanical strength of the final product.

From earlier work in enzyme technology it is known that polymers formed from various acrylic monomers offer a good choice as support for immobilization (19). Most studies reported have used tightly crosslinked polymers. However, if the

material is to be used for affinity purification of macromolecules, large pores in the gel are a prerequisite. Entrapment was therefore performed with a high percentage of the crosslinking agent *N,N'*-methylenebisacrylamide. The preparations obtained showed excellent flow properties and a high yield of immobilized bacterial cells was achieved. As the macromolecules being purified have to enter the interior of the gel beads, bind to the immobilized structures and, on desorption, be transported out of the beads, small particles will improve the separation properties, because of the short distances available for diffusion but this benefit will be achieved only at the expense of the flow properties. To improve the flow rate without loss of any of the advantages offered by small beads, small beads were formed in the dispersion polymerization process. At the end of the polymerization process, the stirring speed was lowered, thereby causing the small sticky beads to aggregate into raspberry-like clusters, called "berry-beads." After appropriate washing these aggregates were packed in chromatographic columns and used in the subsequent experiments.

A problem common to all affinity chromatographic methods has been leakage of the ligand from the support (20, 21). When operating with single molecules covalently bound to a carrier polymer different coupling procedures have been tried until types of bonds stable under the conditions used have been found. In the case of immobilized cells, formation of a stable material may be a more difficult problem, since it is not enough to keep the cells on the support but also detachment of the various entities on the cell surface has to be prevented.

To ensure that no such detachment of cells or surface molecules occurred, immobilized preparations of ^{125}I -labeled cells were prepared and the eluates tested for radioactivity. The fractions containing radioactivity were also examined microscopically. The eluates obtained in subsequent adsorption cycles contained only a low level of radioactivity. However, when the flow was stopped and the immobilized cells remained in contact with 0.2 M glycine-HCl an elevated level of radioactivity was present in the eluate when the flow was resumed. Microscopically, it was seen that a smooth surface of polymer covered the bacterial cells. Thus it was not expected that free cells would be found in the eluate, at least not in the initial cycles. Microscopic examination of the eluates confirmed the absence of whole cells.

As the bacterial strains used are pathogenic, only heat-killed cells were used. In other experiments it has been shown that cells entrapped in a gel matrix still keep their viability (22). Thus, with nonpathogenic bacterial strains it would have been possible to determine any leakage from the column by applying the eluate to agar plates and counting the colonies formed.

Affinity chromatographic materials based on immobilized bacteria are, from many viewpoints, easier to use than those based on immobilized mammalian cells (e.g., red blood cells), since the rigid cell walls of the bacteria are stabilized with covalent bonds, whereas mammalian cells are enclosed by membranes with much looser structures. The stability is reflected in the minimal leakage from the immobilized bacterial cells. Furthermore, when preparing the gel bead dispersion of the water phase containing the bacteria, an organic solvent was used. This procedure did not seem to interfere with the structure of the bacterial cell walls, whereas naked cell membranes are easily destroyed.

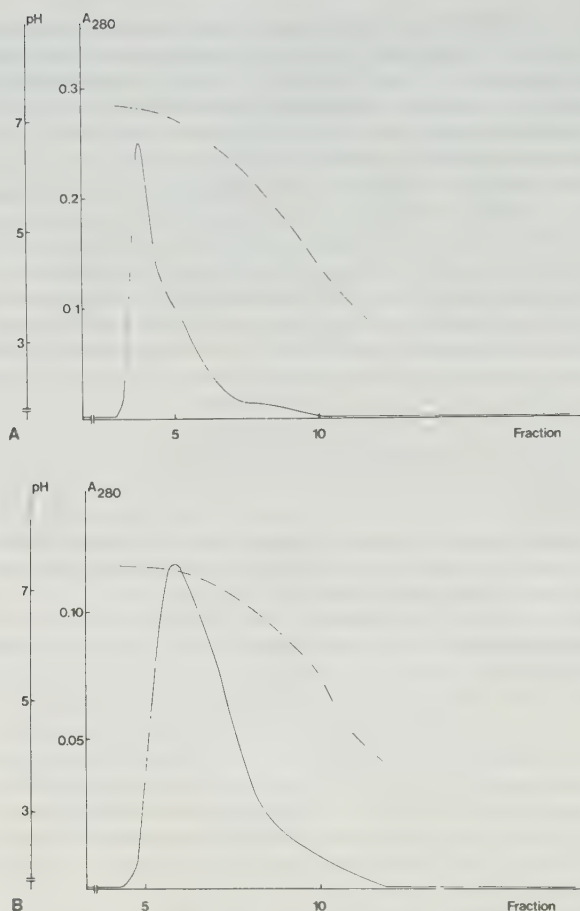


FIG. 1. Elution profiles of proteins from bacterial cells columns consisting of different bacterial strains entrapped in polyacrylamide. A mixture of human serum albumin (0.1 mg/ml) and IgG (0.1 mg/ml) was applied in PBS (2 ml). After washing the column until the eluate showed no absorbance at 280 nm (A_{280} , —), a pH gradient of phosphate-citrate in the pH region 7.0–2.0 was applied (---). (A) Immobilized group A streptococcus AR 1 used as adsorbent material. (B) Immobilized group A streptococcus AW 43 used as adsorbent material. (C) Immobilized group G streptococcus G 148 used as adsorbent material. (D) Immobilized *Staphylococcus aureus* strain Cowan I used as adsorbent material.

Affinity Chromatography of Serum Proteins on Bacterial Cell Columns

Based on the results presented in Table I, efforts were made to produce preparations of bacterial cells entrapped in agarose and, particularly, polyacrylamide for affinity chromatography studies. The bacteria used in these experiments poses the ability to bind some serum proteins (12, 23, 24). The different strains of streptococci and the *S. aureus* strain Cowan I differ in their

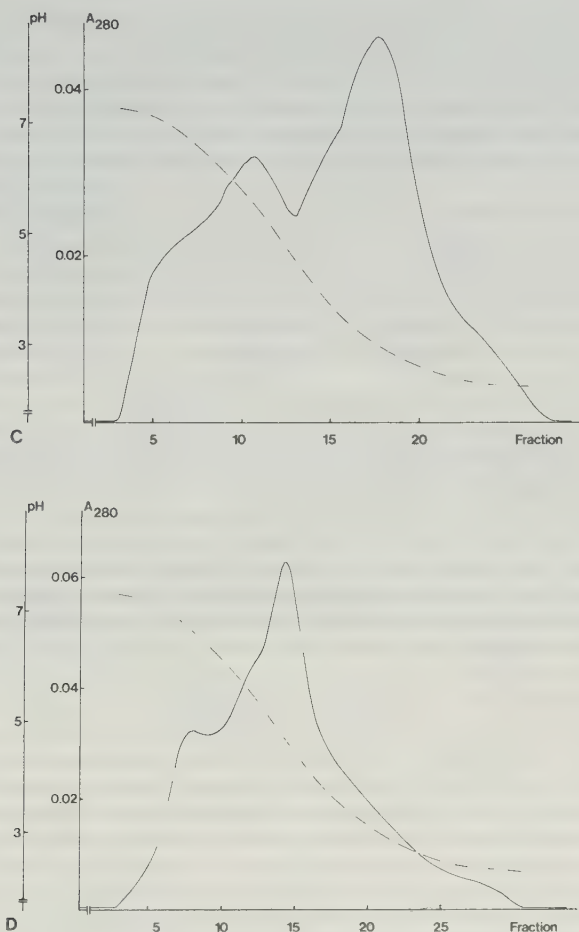


Fig. 1.—Continued

binding of serum proteins and also in their binding strength and capacity. Figure 1 shows elution profiles obtained on chromatography of a mixture of human serum albumin and IgG to columns of immobilized streptococcal strains G-148, AR 1, AW 43 and the *S. aureus* strain Cowan 1 as the affinity material. Elutions were performed with a pH gradient of phosphate-citrate buffer ranging from pH 7.0 to 2.0. It is clear from the elution diagrams that use of the three different streptococcal strains resulted in different elution profiles. Judging from rocket immunoelectrophoretic analysis of the eluates albumin was not bound to either of the two group A strains, in contrast with the group G streptococcus. This phenomenon is in line with binding specificity data reported earlier (23) and with the presence of specific albumin receptors in groups C and G streptococci only (25). Differences in the

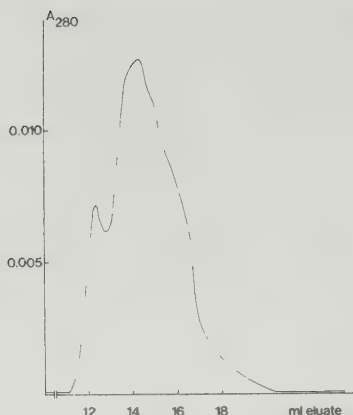


FIG. 2. Elution profile of protein from an immobilized streptococcus G 148 column, when serum was applied to the column. Excess of nonbinding proteins was eluted with PBS and the elution of proteins was performed by a pulse of 0.2 M glycine-HCl, pH 2.2.

elution of IgG were also observed. After an affinity cycle the adsorbent was regenerated by washing with 0.2 M glycine-HCl, pH 2.2. This treatment has been used satisfactorily for column regeneration in analytical applications involving IgG-antigen interaction (26), carbohydrate-lectin interaction (27), and protein A-IgG interaction (28).

In another set of experiments whole serum was applied to the column materials. After subsequent washing until absorbance at 280 nm could not be detected, a pulse of glycine-HCl, pH 2.2, was introduced. The elution pattern is seen in Fig. 2. The procedure was repeated several times, always with the same result. This finding means that the column can be reused several times and that the structures on the cell surfaces responsible for the binding are not modified by the adsorption-desorption process.

Gradient elution of serum proteins adsorbed to immobilized cell columns was also performed. Figure 3 shows typical results. To identify the individual proteins eluted, each fraction having absorbance at 280 nm was analyzed by rocket immunoelectrophoresis against human IgG and human serum albumin, respectively. Figure 4 gives the results of closer study of the fractions obtained in the chromatographic procedure using G-148 that is illustrated in Fig. 3. The A_{280} plot in Fig. 4A gives an indication of the position of protein in the eluate. To corroborate the distribution protein was also determined with the Folin reagent and the results are shown in Fig. 4B. The first peak obtained in the A_{280} profile did not respond to the Folin test and was thus regarded as not being protein. All the fractions were studied immunoelectrophoretically. The results (Figs. 4C and D) showed serum proteins in the last peak, where albumin and immunoglobulin G were found. The immunoelectrophoresis tests were run against rabbit anti-total human serum as well as against specific antisera for the proteins identified in the first run. As mentioned earlier, when studied with 125 I-labeled bacterial cells the leakage of protein from the columns was very low. The hitherto unidentified peak of nonproteinaceous material is now receiving attention in our laboratories.

The binding ability of the columns varied also with the flow rate. This finding indicates the presence of diffusional restriction in the system studied. In some experiments the adsorption process was carried out in a batch procedure and subsequently the gel was packed in a column and washed with buffer before elution. Such experiments did not reveal any new binding functions on the affinity material. A higher degree of binding was observed using this approach than took place in the conventional procedure.

This report illustrates the potential use of affinity chromatography with affinity adsorbent materials based on immobilized cells and organelles. The use of *S. aureus* and streptococcal cells has shown their potential role in the isolation of serum proteins known to bind selectively to these strains. Furthermore, the use of affinity chromatography may also be useful in the study of interactions between cells and macromolecules. Investigations of these problems are now in progress in our laboratories.

In all the column materials used, the amounts of bacterial cells used were identical. When comparing the elution patterns obtained, several differences were observed. Besides variation of the capacity to bind different serum proteins, differences were also observed in strength of binding, i.e., the proteins were eluted at slightly different regions in the elution gradients. Concerning the total binding capacity of the bacterial columns, comparisons of the different preparations were not made. However, the ability of the group G streptococcus strain G-148 to bind albumin (570 $\mu\text{g}/\text{test}$) was calculated to be 16% of that reportedly (23) bound by free bacterial cells. This figure represents a relatively good retention of binding activity. Similar comparisons indicated that the total binding of IgG (140 $\mu\text{g}/\text{test}$) was 6.4% of the capacity of free bacterial cells.

GENERAL DISCUSSION

Affinity chromatographic adsorption materials produced according to the acrylamide entrapment procedure described here offer several advantages over methods on record (8, 9). Virtually no leakage of surface structures from the immobilized cells was observed even after repeated use of the column material. The same gel can be used for months at room temperature provided some bacteriostatic agent is added to the buffer.

Furthermore, no leakage of whole cells was observed. Such stability is particularly valuable in situations where leakage of adsorbed cells might result in contamination of the eluate and to decrease in the capacity of the affinity column. No such drawbacks of the present column material were observed.

The use of immobilized whole cells as affinity adsorbents has certain potential applications. This paper demonstrates the use of bacterial cells with specific surface receptors for serum proteins to selectively bind these serum components. Staphylococci and streptococci have been described as having specific receptors for fibrinogen, IgG, IgA, haptoglobin, albumin, and aggregated β_2 -microglobulin (23). Using bacterial strains with defined surface receptors for the preparation of affinity chromatography column materials, their binding specificities can be used to advantage for purification procedures, for radioassays, or for the removal of proteins present as contaminants.

Human IgG and IgA bind to separate receptors on group A streptococci (23). IgG Fc binding structures show different specificities in various bacterial species, there being at least five major Fc receptor types (12, 24, 25). This spectrum of

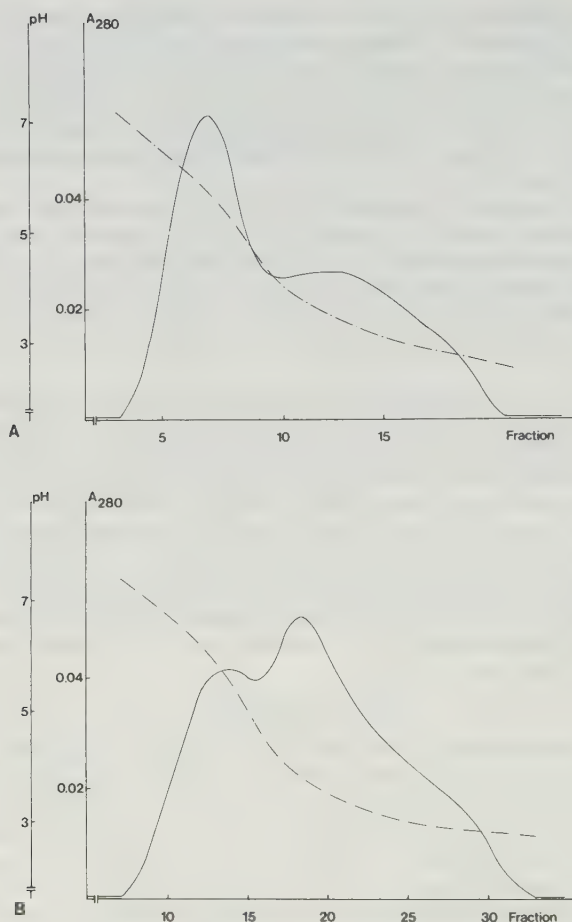


FIG. 3. Elution profiles obtained from the same columns described in Fig. 1, when serum was applied. Experimental details are the same as in the legend to Fig. 1.

immunoglobulin-binding characteristics might provide a basis for new methods for class and subclass separation of human and animal immunoglobulins.

Another potential application of immobilized cells is in adsorption procedures for the preparation of specific antisera. When immunizing an animal with bacterial antigens the crude antiserum cross-reacts with other related bacterial species and types in addition to displaying the expected specificity. In such cases cross-reacting cells may be used in an affinity procedure for adsorbing all cross-reacting antibodies and leaving the non-cross-reacting ones in the antiserum. The possibility of using such columns repeatedly after recycling implies the availability of an inexpensive, stable, high-capacity adsorbent.

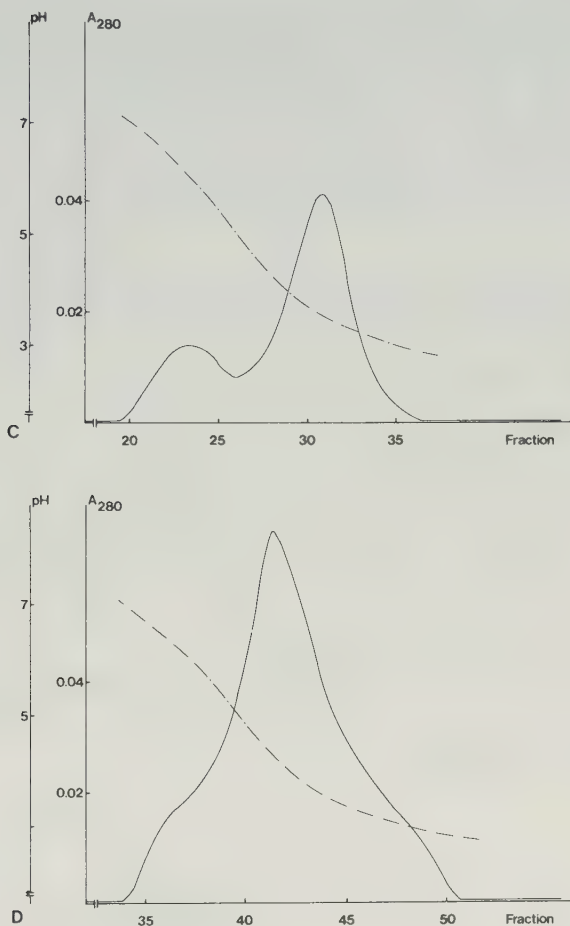


Fig. 3.—Continued

Recently, whole bacterial cells have been used in a new technique aiming at immunotherapy of cancer (29). *Staphylococcus aureus* cells with IgG-binding protein A deplete serum samples of their *in vitro* tumor immunity blocking activity (30). The use of free bacterial cells offers several problems. The column material described here might provide a solution to such problems.

Still other applications of these immobilized cell columns might be envisaged. Their use as affinity adsorbents is not confined to interactions with serum proteins. Thus it has been shown that immobilized cells may be used in the purification of lectins (8, 9). This application might become important as more and more information is collected concerning rare lectins with specificities for carbohydrates consisting of sequences of only a few sugar entities arranged in a very special steric

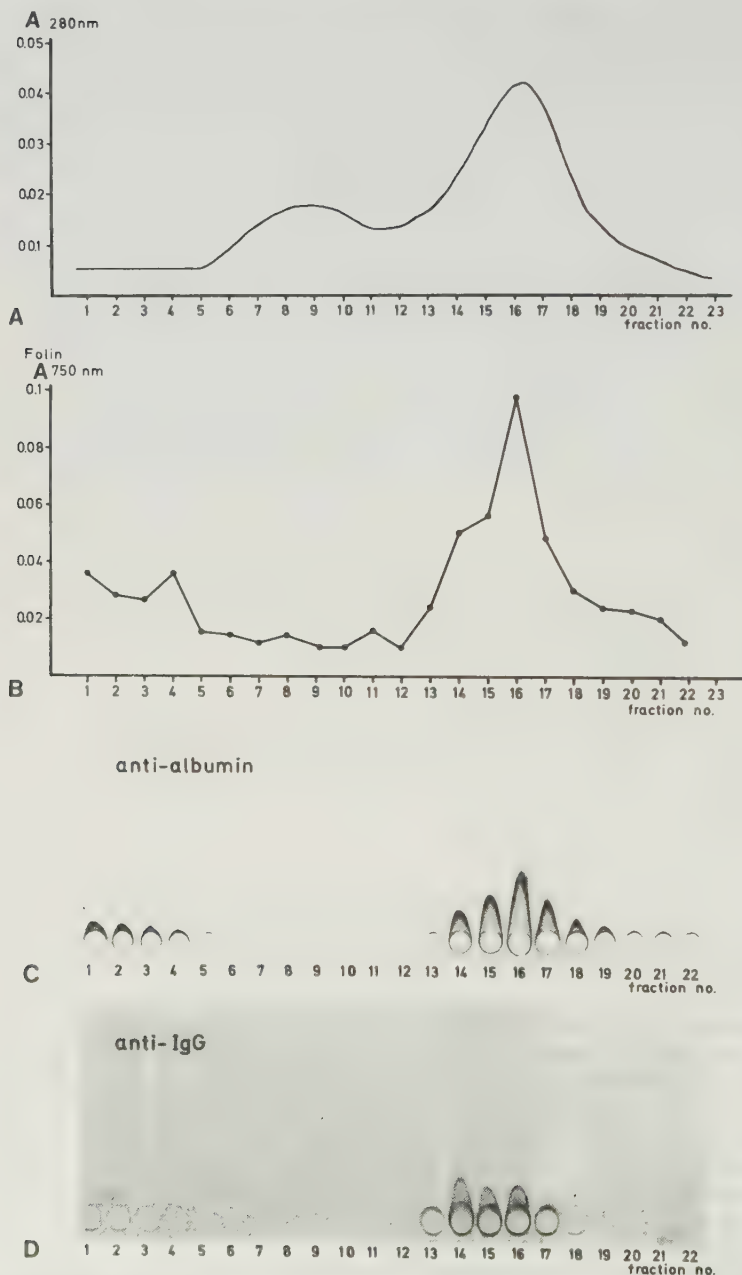


FIG 4. Analyses of the eluate obtained after application of serum to an immobilized *Streptococcus G* 148 column. (A) Absorbance, $A_{280 \text{ nm}}$. (B) Protein distribution, $A_{750 \text{ nm}}$. (C) Immunological reactivity to anti-albumin. (D) Immunological reactivity to anti-IgG.

configuration. In such cases it is extremely difficult to synthesize the ligand and to use conventional affinity chromatographic procedures. Cell column chromatography seems to be an obvious alternative approach.

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REFERENCES

1. JACOBY, W. B. AND WILCHEK, M. (eds.) (1974) *Methods in Enzymology*, Vol. 34, Academic Press, New York.
2. MOSBACH, K. (1978) in *Advances in Enzymology and Related Areas of Molecular Biology*, (Meister, A., ed.), Vol. 46, pp. 205-278, Wiley, New York.
3. EASTERDAY, R. L., AND EASTERDAY, I. (1974) in *Immobilized Biochemicals and Affinity Chromatography* (Dunlap, R. B., ed.), pp. 123-134, Plenum, New York.
4. OHLSSON, R., BRODELIUS, P., AND MOSBACH, K. (1972) *FEBS Lett.* **25**, 234-238.
5. KOCH-SCHMIDT, A.-C., MATTIASSEN, B., AND MOSBACH, K. (1977) *Eur. J. Biochem.* **81**, 71-78.
6. BOORSMA, D. M., AND STREEFKERK, J. G. (1978) in *Immunofluorescence and Related Techniques* (Knapp, W., Holmbar, K., and Wick, G., eds.), pp. 225-235, North-Holland, Amsterdam.
7. LEVY, D. E., AND EVELEIGH, J. W. (1978) *J. Immunol. Methods* **22**, 131-142.
8. MCKINNEY, R. M., THACKER, L., WONG, M. C., AND HEBERT, G. A. (1978) *J. Immunol. Methods* **21**, 1-10.
9. OCHOA, J. L., AND CHRISTIANSEN, T. (1978) *FEBS Lett.* **90**, 145-148.
10. GENAUD, L., GUILLOT, J., DAMEZ, M., AND CONLET, M. (1978) *J. Immunol. Methods* **22**, 339-346.
11. KRONVALL, G. (1973) *J. Med. Microbiol.* **6**, 187-190.
12. KRONVALL, G. (1973) *J. Immunol.* **111**, 1401-1406.
13. DAHLQVIST, A., MATTIASSEN, B., AND MOSBACH, K. (1973) *Biotechnol. Bioeng.* **15**, 395-402.
14. AXÉN, R., PORATH, J., AND ERNBACK, S. (1967) *Nature (London)* **214**, 1302-1304.
15. GERSTEN, D. M., AND MARCHALONIS, J. J. (1978) *J. Immunol. Methods* **24**, 305-309.
16. KIERSTAN, M., AND BUCKE, C. (1977) *Biotechnol. Bioeng.* **19**, 387-397.
17. HIJERTÉN, S. (1964) *Biochim. Biophys. Acta* **79**, 393-398.
18. LAURELL, C.-B. (1972) *Scand. J. Clin. Lab. Invest.* **29**, Suppl. 124, 21-37.
19. KOCH-SCHMIDT, A.-C. (1977) in *Biomedical Applications of Immobilized Enzymes and Proteins* (Chang, T. M. S., ed.), Vol. 1, pp. 47-65, Plenum, New York.
20. TESSER, G. I., FISCH, H. U., AND SCHNYZER, R. (1974) *Helv. Chim. Acta* **57**, 1718-1730.
21. MÄNSSON, M.-O., MATTIASSEN, B., GESTRELIUS, S., AND MOSBACH, K. (1976) *Biotechnol. Bioeng.* **18**, 1145-1159.
22. MATTIASSEN, B., LARSSON, P.-O., AND MOSBACH, K. (1977) *Nature (London)* **268**, 519-520.
23. KRONVALL, G., SIMMONS, A., MYHRE, E. B., AND JONSSON, S. (1979) *Infect. Immun.* **25**, 1-10.
24. MYHRE, E. B., AND KRONVALL, G. (1977) *Infect. Immun.* **17**, 475-482.
25. MYHRE, E. B., AND KRONVALL, G. (1980) *Infect. Immun.* **27**, 6-14.
26. MATTIASSEN, B., BORREBAECK, C., SANFRIDSSON, B., AND MOSBACH, K. (1977) *Biochim. Biophys. Acta* **483**, 221-227.
27. MATTIASSEN, B., AND BORREBAECK, C. (1978) *FEBS Lett.* **85**, 119-123.
28. MATTIASSEN, B., AND BORREBAECK, C. (1978) in *Enzyme Labelled Immunoassay of Hormones and Drugs* (Pal, S. B., ed.), pp. 91-105, de Gruyter, Berlin.
29. BANSAL, S. C., BANSAL, B. R., THOMAS, H. L., SIEGEL, P. D., RHOADS, J. E., JR., COOPER, D. R., TERMAN, D. S., AND MARK, R. (1978) *Cancer* **42**, 1-18.
30. STEELE, G., JR., ANKERST, J., AND SJÖGREN, H. O. (1974) *Int. J. Cancer* **14**, 83-92.

II

Isolation and Partial Characterization of a Substance from Carrots, *Daucus carota*, with Ability to Agglutinate Cells of *Streptococcus mutans*¹

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Key Words. Affinity chromatography · Agglutination · Dextran · *Streptococcus mutans*

Abstract. A substance found in carrot, *Daucus carota*, with the ability to agglutinate *Streptococcus mutans* cells, was purified by means of affinity chromatography on immobilized whole *S. mutans* cells. The agglutinin was further characterized in order to determine the nature of the participating molecular entities in the binding reaction. The agglutinin was shown to be heat and trypsin stable, whereas treatment with dextranase destroyed its ability to agglutinate *S. mutans* cells. The cells, however, could be agglutinated after treatment with dextranase, but could not be agglutinated after treatment with heat or trypsin. The agglutination of *S. mutans* cells with carrot agglutinin was competitively inhibited by low molecular weight dextran. It was concluded that protein structures on the cell surface of *S. mutans* were essential for the binding of the carrot agglutinin. Furthermore, the agglutinin, isolated from *D. carota*, seemed to be carbohydrate in nature, presumably a dextran.

Many studies have described factors that regulate the occurrence of *Streptococcus mutans* in the oral cavity. Among factors endogenous to the host, protective systems such as salivary immunoglobulins and agglutinins have been of a special interest.

Sucrose has received the most attention among exogenic factors because it acts as a substrate for synthesis of adhesive glucans which enhance colonization. Some exogenous materials may have certain effects which resemble those of the agglutinins [Hamada et al., 1977]. For example, carrot, *Daucus carota*, has been shown to contain a substance that agglutinates *S. mutans* cells [Bratthall, 1978]. This paper reports on further studies of the agglutinating substance from *D. carota*.

¹ Part of this investigation was presented at the VIIIth International Symposium on Streptococci and Streptococcal Diseases, Lund, Sweden, 1981. The study was supported by The Swedish Medical Research Council, Project No. 5999.

Materials and Methods

Chemicals Used

Acrylamide, N,N'-methylene-bis-(acrylamide), N,N,N',N'-tetramethylethylenediamine and ammoniumperoxodisulfate, all of analytical grade, were obtained from Merck, Darmstadt, FRG. Arlacel 83 was purchased from Atlas Chemie, FRG, trypsin, type III, and soybean trypsin inhibitor from Sigma Chemical C., St. Louis, Mo, USA, dextranase from CSR, UK. and Sepharose 4B, Dextran T10 and Dextran T70 from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of analytical grade.

Bacterial Cells

S. mutans, strain OMZ 65, serotype g, obtained from cultures on blood agar plates, was inoculated in pre-dialyzed tryptone-yeast medium according to Carlsson et al. [1969]. After incubation for 20 h in 95% N₂ and 5% CO₂, the cells were harvested by centrifugation at 1,860 g for 10 min. The cells were washed twice with phosphate-buffered saline (0.10 M Na₂HPO₄/NaH₂PO₄, 0.15 M NaCl, pH 7.0; PBS), suspended and finally stored in the same buffer containing 0.05% sodium azide. A standardized cell suspension, approximately 10% (w/v), was obtained by diluting the washed cells with PBS.

Carrot Extract

Carrot extract was prepared from well-rinsed Swedish carrots, *D. carota*, with the outer layer removed. The carrots were cut in small pieces, mixed with twice their weight of PBS and homogenized in a Waring blender for 5 min. The homogenate was left at 4 °C for 1 h. The homogenate was filtered and centrifuged for 10 min at 16,000 g and the clear supernatant obtained was stored at -20 °C.

Preparation of Affinity Sorption Material Containing Immobilized *S. Mutans* Cells

The immobilization of *S. mutans* cells followed a method previously described [Mattiasson et al., 1980]. 100 ml of a hydrophobic phase, consisting of toluene and chloroform (2.64/1.00, v/v), including 0.7 ml of a surfactant medium, Arlacel 83, was slowly stirred in a 500-ml Erlenmeyer flask under nitrogen gas for 30 min. A cell suspension, obtained by mixing 3.4 ml of the standardized *S.*

mutans cell suspension (approx. 10% (w/v) and PBS to a final volume of 10.4 ml at 4 °C, was mixed with 4.2 ml monomer solution, containing 1.17 g acrylamide and 0.456 g N,N'-methylene-bis-(acrylamide). This cell-containing hydrophilic phase was bubbled with nitrogen gas for 3 min prior to the addition of a catalyst system.

The polymerization process was initiated by the addition of 50 µl N,N,N',N'-tetramethylethylenediamine and 75 mg ammoniumperoxodisulfate, dissolved in 1 ml PBS. The stirring speed of the nitrogen-treated hydrophobic phase was increased before the cell-monomer suspension was rapidly poured into the hydrophobic phase. The hydrophilic phase, containing cells, thereby formed small droplets in the hydrophobic phase. Upon polymerization, solid particles were formed within 10–15 min. The stirring speed of the reaction mixture was at this moment decreased in order to let the small beads, which were sticky on their surfaces due to uncomplete polymerization, aggregate and form raspberry-like clusters. After 30 min, when the polymerization process was completed, the hydrophobic phase was decanted and the solid material was washed with large portions of luke-warm water containing small amounts of detergent.

Affinity Chromatographic Purification of the Substance from *D. carota* Interacting with Immobilized *S. mutans* Cells

The polyacrylamide-entrapped cell preparation was suspended in PBS and packed in a column, 1.8 × 10.0 cm, equipped with flow adaptors. A constant flow of buffer, PBS, was pumped through the column by means of a peristaltic pump. The effluent was passed through an UV detector (Uvicord S, LKB Wallac, Bromma, Sweden), for continuous measurement of the absorbance at 280 nm. When the column was equilibrated, 1 ml of crude carrot extract, diluted with 1 ml PBS, was applied to the column. The material not binding to the column was washed out with PBS. After regaining zero absorbance in the effluent, the bound material was eluted by changing the buffer from PBS, pH 7.0, to 0.2 M glycine-HCl, pH 2.2. The eluted material was collected in fractions of 2.5 ml and each fraction was extensively dialyzed towards three changes of PBS, and then concentrated to a final volume of 0.5 ml. The fractions were finally stored at 4 °C.

Determination of Agglutinating Titer

In order to determine the ability of the purified fractions to agglutinate *S. mutans* cells, a simple analysis of the dialyzed and concentrated fractions was performed. This was accomplished by mixing a 2% w/v suspension of the oral streptococci with an equal volume of serial twofold dilutions of each of the purified fractions. After 12 h incubation in polyethylene tubes at room temperature, each incubation mixture was examined for agglutination using a light microscope.

Immobilization of Soybean Trypsin Inhibitor

40 g wet weight of Sepharose 4B was washed with distilled water on a porous glass filter. The gel was activated with 3 g CNBr dissolved in 50 ml distilled water for 5 min [Axen et al., 1967], the pH of which was kept at 10.5 by the dropwise addition of a 2 M NaOH solution. The activated gel was washed repeatedly with ice-cooled 0.1 M NaHCO₃ on a porous glass filter and then incubated with 120 mg soybean trypsin inhibitor, dissolved in 40 ml 0.1 M NaHCO₃, for 12 h at 4 °C. After coupling, the gel was extensively washed with 0.1 M NaHCO₃, followed by 1 M NaCl and 1 M glycine-NaOH, pH 9.0, and then suspended for 1 h in 1 M sodium acetate and finally washed with cold distilled water. The gel material was stored with 0.2% sodium azide added to the perfusion buffer packed in a glass column.

Treatment of Purified Carrot Extract

Trypsin Treatment. 400 μ l purified agglutinin, with an agglutinating titer of 1:128, was incubated with 10.0 mg trypsin, 10,200 BAEE units/mg protein, for 1 h at room temperature. Trypsin was removed from the mixture by affinity chromatography on soybean trypsin inhibitor coupled to Sepharose 4B. The treated sample was recovered in the void volume and concentrated to the initial volume, 400 μ l.

Heat Treatment. 400 μ l purified agglutinin, titer 1:128, was heated in a water bath at 90 °C for 20 min and the solution was then cooled to room temperature.

Dextranase Treatment. 400 μ l purified agglutinin, titer 1:128, was incubated with 10.0 mg dextranase, 20,000 units/mg protein, for 12 h at room temperature. Dextranase was removed, prior to the agglutination study, by heat inactivation at 80 °C for 10 min.

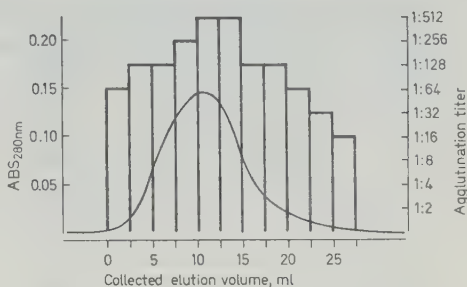


Fig. 1. Elution profile of carrot agglutinin from affinity sorption material containing immobilized whole cells of *S. mutans* OMZ 65.

Treatment of *S. mutans* Cells

The treatment of the bacterial cells with trypsin, heat and dextranase followed the methods used for treatment of purified agglutinin as described above. The treated cells were recovered by centrifugation. The agglutinating titer for the treated agglutinin and bacterial cell suspensions was determined as earlier described.

Agglutination Inhibition by Dextran

In order to determine whether the *S. mutans* cell surface receptor for dextran is identical with the structure binding to the carrot agglutinin, an inhibitory assay was carried out. 200 μ l suspension of *S. mutans* was incubated with 100 μ l carrot agglutinin and 100 μ l solution of dextran T10 (0.1% w/v). The assay was also carried out with dextran T70. The samples were examined after 12 h for agglutination.

Results

Affinity Chromatography Purification of Carrot Agglutinin

It has been demonstrated that surface structures on immobilized whole cells can be used for affinity chromatographic purification [Mattiasson and Ramstorp, 1980]. The result of the purification process is shown in figure 1. The elution of carrot agglutinin bound to the immobilized *S. mutans*

Table I. Effects of various treatments of purified carrot agglutinin on the agglutination of *S. mutans* OMZ 65

Aggl. titer	Agglutinin treated with			Un-treated
	heat 90°C	trypsin	dextranase	
1:2	+	+	-	+
1:128	+	+	-	+

Table II. Effects of various treatments of *S. mutans* OMZ 65 cells on the agglutination by carrot agglutinin

Aggl. titer	Cells treated with			Un-treated
	heat 90°C	trypsin	dextranase	
1:2	-	-	+	+
1:128	-	-	+	+

cells is presented as agglutination activity in the eluate and a corresponding increase in absorbance.

Effects on Agglutination of Various Treatments of Agglutinin and Bacterial Cells

Trypsin-treated purified carrot agglutinin gave, when incubated with untreated *S. mutans* cells, an agglutination pattern similar to that obtained when incubating native carrot agglutinin with untreated cells. The same result was obtained when heat-treated carrot agglutinin was incubated with untreated cells while the dextranase-treated agglutinin completely lost the agglutinating activity (table I). This sensitivity to dextranase treatment was also shown to be true for the crude extract.

In order to collect information concerning the structures on the cell surface of *S.*

mutans involved in agglutination, the cells were treated with trypsin, heat or dextranase. When incubating trypsin- or heat-treated cells with native agglutinin, no agglutination appeared. In contrast, dextranase-treated cells remained unaffected (table II).

Agglutination Inhibition by Dextran

The reaction between *S. mutans* cells and carrot agglutinin, leading to agglutination of the cells, was completely inhibited by the addition of dextran T10 but no effect on agglutination was observed when dextran T70 was added.

Discussion

Earlier demonstration of the high specificity of the carrot extract suggested the agglutinin to be of lectin nature, although these substances are more commonly found in plant seeds and not in the vegetative parts of the plant [Lis and Sharon, 1981]. The fact that the agglutinating substance was shown to be heat stable and not affected by trypsin diminishes this possibility, since lectins at least partly consist of proteins. The total loss of activity after dextranase treatment indicated that the agglutination is induced by a polysaccharide extracted from the carrot.

Agglutination of *S. mutans* cells by dextrans of microbial origin has been the subject of many investigations. The most effective dextrans for agglutination are those of high molecular weight (2×10^6) and this agglutination has been reported to be inhibited by low molecular weight dextrans (10^4) [Gibbons and Fitzgerald, 1969; McCabe et al., 1976], although a contradictory report on this point exists [Wu-Yan et al., 1978].

In this study, the inhibitory effect of incubating the cells with low molecular weight dextran indicates that the agglutinating substance utilizes a dextran binding site on the cell surface. The exact nature of this structure has been much disputed but it has been shown to be sensitive to trypsin [Spinell and Gibbons, 1974; Kelstrup and Funder-Nielsen, 1974], dextranase [Wu-Yan et al., 1978] and, with one exception [McCabe and Smith, 1975], to heat [Gibbons and Fitzgerald, 1969].

The findings in this study coincide as to the trypsin and heat sensitivity but the agglutination of dextranase-treated cells is unaffected. A possible explanation for this is that the dextran-mediated agglutination involves a binding site composed of both polysaccharide and protein, but the agglutinating substance from carrot binds only to the protein part of the structure. Another explanation is that the dextranase, reported to inactivate the binding site for dextran [Wu-Yan et al., 1978], was not free from protease activity.

It is possible that the agglutinin derived from carrot is but one example of a widespread dietary content of agglutinating substances. If so, the influence of such substances on the ecology of the oral microflora should not be overlooked.

References

Axen, R.; Porath, J.; Ernback, F.: Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. *Nature, Lond.* 214: 1302-1304 (1967).

Bratthall, D.: *Daucus carota* (carrot). A selective bacteriosorbent; in *Secretory immunity and infection*, pp. 327-333 (Plenum Press, New York 1978).

Carlsson, J.; Newburn, E.; Krasse, B.: Purification and properties of dextransucrase from *Streptococcus sanguis*. *Archs oral Biol.* 14: 469-478 (1969).

Gibbons, R.J.; Fitzgerald, R.J.: Dextran-induced agglutination of *Streptococcus mutans*, and its potential role in the formation of dental plaques. *J. Bact.* 98: 341-346 (1969).

Hamada, S.; Gill, K.; Slade, H.D.: Binding of lectins to *Streptococcus mutans* cells and type-specific polysaccharides and effect on adherence. *Infect. Immunity* 18: 708-716 (1977).

Kelstrup, J.; Funder-Nielsen, T.D.: Adhesion of dextran to *Streptococcus mutans*. *J. gen. Microbiol.* 81: 485-489 (1974).

Lis, H.; Sharon, N.: Lectins in higher plants; in *The biochemistry of plants*, vol. 6, pp. 371-447 (Academic Press, New York 1981).

Mattiasson, B.; Ramstorp, M.: Affinity chromatographic purification of proteins using immobilized cells; in *Enzyme engineering*, vol. 5, pp. 401-403 (Plenum Press, New York 1980).

Mattiasson, B.; Ramstorp, M.; Widebäck, K.; Kronvall, G.: Affinity chromatography on immobilized bacterial cells with receptors for human serum proteins. *J. appl. Biochem.* 2: 321-335 (1980).

McCabe, M.; Haynes, A.U.; Hamelik, R.M.: Cell adherence of *Streptococcus mutans*; in *Microbial aspects of dental caries*, pp. 413-424 (Information Retrieval, Washington 1976).

McCabe, M.; Smith, E.: Relationship between cell-bound dextran-sucrase and the agglutination of *Streptococcus mutans*. *Infect. Immunity* 12: 512-520 (1975).

Spinell, D.M.; Gibbons, R.J.: Influence of culture medium on the glycosyltransferase and dextran binding capacity of *Streptococcus mutans* 6715 cells. *Infect. Immunity* 10: 1448-1451 (1974).

Wu-Yan, C.D.; Tai, S.; Slade, H.D.: Dextran/glucan binding by *Streptococcus mutans*. The role of molecular size and binding site in agglutination; in *Secretory immunity and infection*, pp. 737-749 (Plenum Press, New York 1978).

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III

APPLICATION OF PARTITION AFFINITY LIGAND ASSAY (PALA) IN A QUICK TEST FOR QUANTITATION OF *STAPHYLOCOCCUS AUREUS* BACTERIAL CELLS

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A novel immunochemical method for the quantitation of bacterial cells is described. The method, which is based on separation of bound from free reactants in aqueous two-phase systems has been studied both as direct and competitive binding assays, with a system consisting of intact cells of *Staphylococcus aureus* and human immunoglobulin G molecules.

To achieve high resolution, one of the reactants was modified so that the two reactants, the bacterial cells and the immunoglobulin molecules, occurred in different phases of the phase system. When binding takes place, the partition of one of the reactants is changed. The degree of change is then correlated to the amount of reactant present.

Using this method, cell numbers in the region 10^5 – 10^7 can be quantified. An assay takes 40–90 min.

INTRODUCTION

Quick diagnostic methods arouse great interest in microbiology, since successful treatment of a disease often depends on early recognition of the condition. Many of the typing procedures routinely used at present are, however, very time-consuming. Immunological techniques have proved both quick and sensitive and various approaches using specific immunological interactions in microbiological assays have been described (Danielsson and Kronvall, 1974; Hildebrand, 1979; Overby and Muskahwar, 1979).

This paper reports a binding assay for the quantitation of bacterial cells. The assay is based on interactions between surface structures on intact cells and specific binding molecules. In the system studied the interaction between protein A on the cell surface of *Staphylococcus aureus* strain Cowan I and human IgG molecules was used. The assay was performed in homogeneous solution and bound and free reactants were separated from each other using aqueous two-phase systems containing polymer/polymer or polymer/salt. These phase systems separate macromolecules, organelles and cells (Albertsson, 1971; Albertsson, 1978; Eriksson and Johansson, 1979) according to their individual surface properties.

Partitioning in aqueous two-phase systems was not used for competitive analysis of macromolecules before the introduction of heavily modified separator molecules (Mattiasson, 1980a). In a binding assay where two reactants are interacting, successful analysis requires that the two reactants are partitioned to different phases in the aqueous two-phase system. This situation may occur spontaneously, especially when assaying small molecules (Mattiasson and Ling, 1980), but otherwise one of the two reactants has to be modified so as to show a predilection for one or the other of the two phases, while the other reactant remains in the other phase. The analytical technique involving this combination of phase separation and competitive binding using separator molecules has been called Partition Affinity Ligand Assay (PALA) (Mattiasson, 1980a; Mattiasson and Ling, 1980).

The present report concerns the application of the PALA-principles in microbiological assays, here exemplified by the system *Staphylococcus aureus* and human immunoglobulin G, which besides being of interest per se may also be regarded as a model system for antigen-antibody reactions.

MATERIALS AND METHODS

Polyethylene glycols (PEG) of molecular weights 4000 and 6000 respectively, and triazine were obtained from British Drug House, Poole, U.K. Monomethoxypolyethylene glycol (M-PEG) 5000 (Carbowax) was a generous gift from Union Carbide, New York, U.S.A. Magnesium sulphate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, was from Merck, Darmstadt, F.R.G., Dextran T 250 from Pharmacia Fine Chemicals, Uppsala, Sweden, triethanolamine-HCl from Fluka AG, Buchs, Switzerland and immunoglobulin G (IgG) from KABI, Stockholm, Sweden. All other chemicals used were of analytical grade.

Heat-treated and formaldehyde-stabilized cells of *Staphylococcus aureus*, strain Cowan I (Kronvall and Frommel, 1970), was a generous gift from Pharmacia Diagnostics, Uppsala, Sweden.

Modification of the reactant with monomethoxypolyethylene glycol

Monomethoxypolyethylene glycol was activated with triazine according to Abuchowski et al. (1976). M-PEG-modified IgG was obtained by adding 20 mg activated M-PEG to a 1 ml solution of 8 mg IgG in 0.1 M triethanolamine-HCl, 1.0 M NaCl (pH 9.4). The mixture was agitated for 1 h at room temperature and finally stored at +4°C.

The *Staphylococcus* cells were modified by adding 2 mg activated M-PEG to 3×10^9 cells, in 1 ml 0.1 M triethanolamine-HCl, 1.0 M NaCl (pH 9.4). The cell suspension was kept under agitation at room temperature for 1 h and finally stored at +4°C.

Radiolabelling of human immunoglobulin G

Human immunoglobulin G was radiolabelled with 1 mCi of ^{125}I per mg protein by the lactoperoxidase method (Thorell and Johansson, 1971). The yield obtained was 91%.

Phase systems

Two different aqueous two-phase systems were used, containing polymer/polymer and polymer/salt, respectively. The polymer/polymer phase system was obtained by mixing 450 μ l 15% (w/w) Dextran T 250 and 450 μ l 10% (w/w) polyethylene glycol 6000, and the polymer/salt system was obtained by mixing 1 ml 30% (w/w) polyethylene glycol 4000 and 3 ml 30% (w/w) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

Binding assays

Direct binding assay. In a direct binding assay of *Staphylococcus aureus*, a sample containing native bacterial cells and a predetermined number of PEG-derivatized ^{125}I -labelled IgG molecules was diluted to a total volume of 100 μ l by addition of 0.1 M phosphate buffer (pH 7.00). The mixture was stirred on a Vortex type mixer and left for 30 min at room temperature. The mixture was then subjected to partitioning in a polymer aqueous two-phase system obtained by mixing 450 μ l 10% (w/w) polyethylene glycol 6000 and 450 μ l 15% (w/w) Dextran T 250. The sample was mixed thoroughly and the phases were allowed to separate for 2 h at room temperature, after which a 200 μ l sample from the top phase was removed. Radioactivity was measured in a 1275 Minigamma, LKB Wallac (Bromma, Sweden) gamma counter.

Competitive binding assay. In a competitive binding assay of *Staphylococcus aureus* native bacterial cells compete with PEG-derivatized cells for a limiting number of ^{125}I -labelled IgG molecules.

A sample containing native *Staphylococcus* cells are mixed with a fixed number of PEG-derivatized cells and a predetermined number of ^{125}I -labelled IgG molecules. The total volume of the system of each sample was kept constant at 100 μ l by addition of 0.1 M phosphate buffer (pH 7.00). The mixture was thoroughly stirred on a Vortex type mixer and left for 30 min at room temperature. The mixture was then subjected to partitioning in a polymer aqueous two-phase system obtained as described in the case of the direct binding assay. A 200 μ l sample from the top phase was taken out for activity measurements in a gamma counter.

RESULTS AND DISCUSSION

The principle of using aqueous two-phase systems to separate bound from free reactant is schematically illustrated in Fig. 1. By modifying IgG with M-PEG the conjugate becomes more hydrophobic and shows a predilection for the PEG-rich top phase (Fig. 1a). When *Staphylococcus* cells are introduced into the system, IgG will be found in increasing amounts in the bottom phase since the IgG interacts with protein A on the cell surface and the cells are per se partitioned to the bottom phase (Fig. 1b).

The binding of a small amount of M-PEG-IgG molecules to the cells does not affect the partitioning of the cells in the system. If, however, there is a

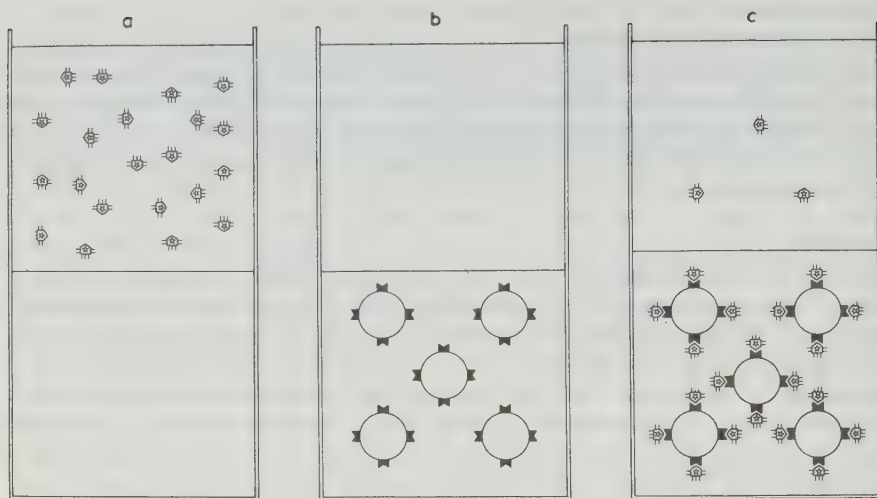


Fig. 1. Schematic representation of direct binding assay of *Staphylococcus aureus*. The cells are incubated with M-PEG-modified ^{125}I -labelled IgG and after 15 min bound IgG is separated from free IgG by partition in an aqueous two-phase system. The phase system consisted of PEG 4000 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mM sodium phosphate (pH 7.00). a: M-PEG-modified ^{125}I -labelled IgG partitioned mainly to the PEG-rich top phase. b and c: native *Staphylococcus aureus* cells partitioned mainly to the salt-rich bottom phase even in the presence of M-PEG-modified IgG and caused a drop in activity of the top phase.

large excess of M-PEG-IgG molecules in the system, the cells will be transported to the upper phase. The other extreme would be when there is an excess of binding positions on the cell surface so that virtually all M-PEG-IgG molecules will be bound and partitioned to the bottom phase. These two extreme situations limit the operational concentration range of the system.

An important step in the analytical procedure is the use of a proper degree of modification of the IgG molecule to make it partition to the PEG-rich phase without inhibiting the interaction with protein A on the cell surface of *Staphylococcus aureus*. To optimize the partition a series of M-PEG-IgG preparations with varying degrees of modification were prepared. Fig. 2 shows that a rather mild degree of derivatization gave an optimal effect. This optimal degree of derivatization was used in all subsequent experiments.

Fig. 3 gives a calibration curve from experiments with M-PEG-modified ^{125}I -labelled IgG molecules. Here the amount of radioactivity in the top phase is plotted as a function of the number of cells added to the reaction mixture.

It was expected from the competitive assay that an even more sensitive assay could be set up. However, a rough calculation of the counts available as iodine-labelled IgG for the assay suggested that a limiting factor in these

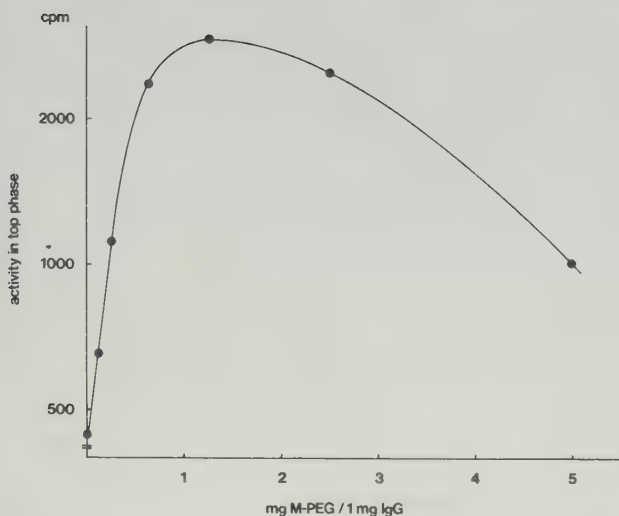


Fig. 2. Determination of the optimal degree of M-PEG-modification of IgG in a direct binding assay of *Staphylococcus aureus*. The diagram shows the activity in the top phase as a function of mg M-PEG added per mg IgG. The experimental conditions were: a predetermined number of *Staphylococcus aureus* cells were incubated for 30 min with a predetermined amount of ^{125}I -labelled IgG molecules modified with various amounts of M-PEG. The incubation mixture was allowed to partition for 30 min in an aqueous two-phase system as described in the legend to Fig. 1. A 400 μl sample was taken from the top phase for activity measurement.

assays is the rate of isotope disintegration. If a lower level of cpm in an assay is set at 200–300 in the phase analyzed, a theoretical lower level of detection may be calculated. The IgG preparation used in these experiments contained approximately 0.5 ^{125}I per IgG molecule. The rate of disintegration of the radioactive isotope is such that in each assay approximately 10^{11} ^{125}I must be present to give 200 cpm. Since the surface of each *Staphylococcus aureus* cell has approximately 10^6 binding sites for IgG (Kronvall and Frommel, 1970), a lower limit will lie in the region of 10^5 cells. The use of more highly labelled IgG may improve the sensitivity of the assay, but only marginally.

It has been argued that for several reasons enzyme labels are preferable to radioactive isotopes (Wisdom, 1976). Such arguments have been the medical hazard involved in the handling of the radioactivity, the short shelf-life of the labelled reagents and the risk that gamma-radiation may destroy vital structures of the labelled molecule (Wisdom, 1976; Mattiasson and Borrebaeck, 1980). Still another argument can now be added, namely the limiting sensitivity. If enzyme labels are used, even small numbers of labelled molecules can be quantified and, from a theoretical point of view,

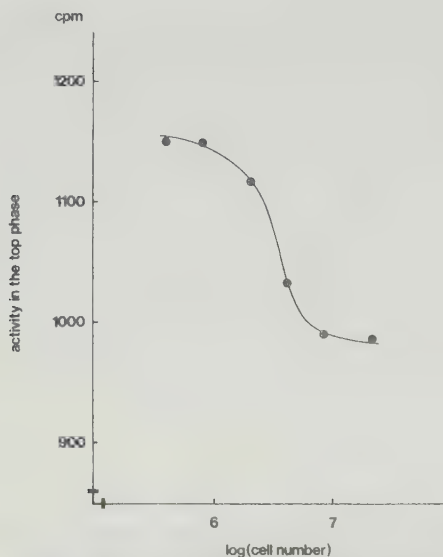


Fig. 3. Calibration curve for *Staphylococcus aureus* obtained in a direct binding assay. The diagram shows the activity in the top phase as a function of the number of *Staphylococcus aureus* cells present in the sample. A fixed amount, 1.2×10^{-13} mol of M-PEG-derivatized ^{125}I -labelled IgG molecules were incubated for 30 min with various numbers of native *Staphylococcus aureus* cells. The incubation mixture was then partitioned for 30 min in an aqueous two-phase system as described in the legend to Fig. 1. A 200 μl sample was taken from the top phase for activity measurement.

the sensitivity of the assay is improved. Preliminary studies using enzyme-labelled reagents show that a higher sensitivity of such an assay is possible.

When using M-PEG-modified IgG molecules as separators, the operating concentration range expressed as the number of cells per ml that can be measured is governed by the intricate balance between too many or too few binding sites. If the number of cells is too small, all binding sites will be occupied and thus no resolution can be expected. On the other hand, if the number of separator molecules bound per cell is increased, the affinity for the bottom phase of the cell will be counteracted by the affinity for the top phase of the modified IgG, with the result that the cell-IgG-M-PEG complex will stay in the interface or, when high binding has taken place, the cells will stay in the top phase. At the other extreme where such an analysis is applicable, an excess of cells will tend to bind all IgG-M-PEG molecules and no differentiation between the different cell counts can be expected.

The two different approaches to bacterial cell quantitation presented here will operate well within concentrations down to 10^6 cells per ml. However, owing to the potential complications with enrichment of cells at the interface, the competitive assay approach may be preferred.

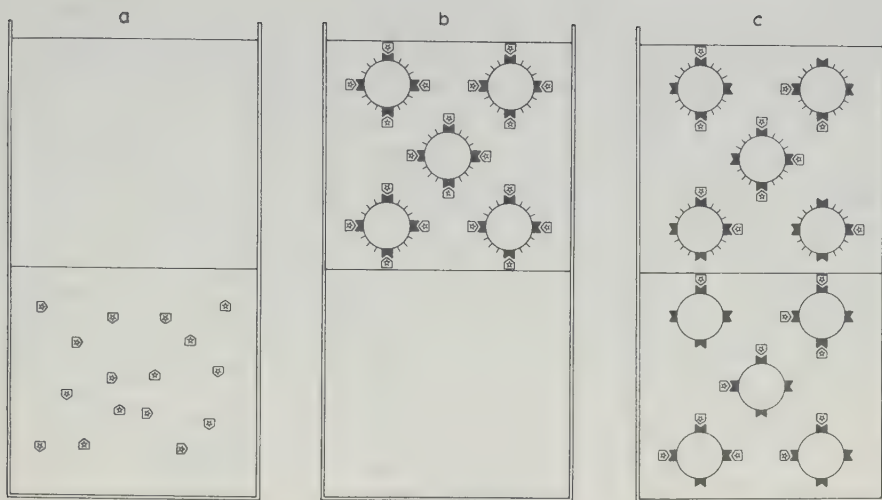


Fig. 4. Schematic representation of a competitive binding assay of *Staphylococcus aureus* where a limited number of ^{125}I -labelled IgG molecules binds to native and M-PEG-modified *Staphylococcus aureus* cells respectively. a: ^{125}I -labelled IgG partitioned mainly to the salt-rich bottom phase. b: M-PEG-modified *Staphylococcus aureus* cells partitioned mainly to the PEG-rich top phase. IgG bound to the cells were thereby transported to the top phase. c: Native *Staphylococcus aureus* cells partitioned mainly to the bottom phase. Since competition between native and M-PEG-derivatized bacterial cells for ^{125}I -labelled IgG takes place in the incubation mixture, a shift in the distribution of ^{125}I -labelled IgG occurs on addition of native cells.

In earlier studies (Mattiasson, 1980a, b; Mattiasson and Ling, 1980), when soluble reactants were used, separation was quick and sensitivity high. This could also be achieved in cell analysis provided whole cells are used as transporters of small marker molecules across the interface.

Most work on immunological analysis in microbiological systems has so far dealt with soluble antigens from the microorganism. It would be easier to analyse such a system, than a system containing whole cells, using the PALA technique. There is no reason to doubt the possibility of a mixed analysis of cells plus free antigens.

In the competitive assay studied, a fixed number of M-PEG-derivatized cells of *Staphylococcus aureus* are competing with various numbers of native cells for a limited number of ^{125}I -labelled IgG molecules. The principle of the competitive binding assay using the PALA method is shown in Fig. 4. To assess the capacity of PEG-modified cells of *Staphylococcus aureus* to transport ^{125}I -labelled IgG molecules to the other side of the interface, a simple titration test was performed. Varying amounts of ^{125}I -labelled IgG molecules were added to a constant amount of M-PEG modified *Staphylococcus aureus* cells. After binding followed by separation in the phase

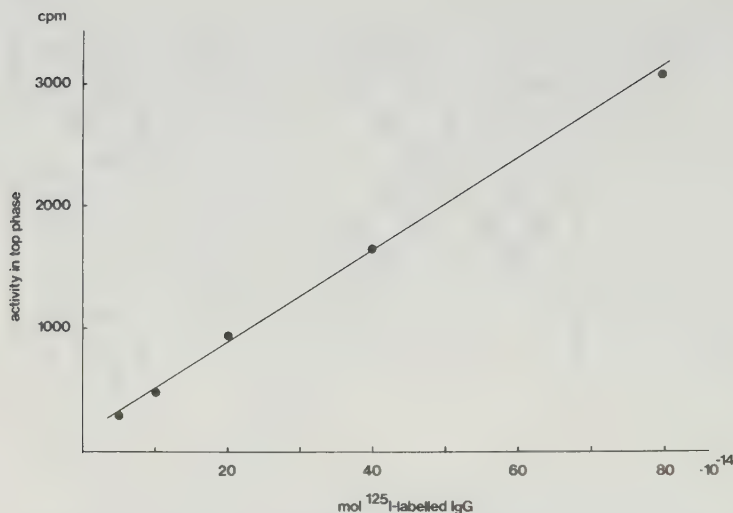


Fig. 5. Titration curve obtained by direct titration of a constant number 6×10^7 of M-PEG-derivatized *Staphylococcus aureus* cells with ^{125}I -labelled IgG. The diagram shows the activity in the top phase obtained when increasing amounts of ^{125}I -labelled IgG were incubated with the cells. After 30 min incubation the mixture was allowed to separate in an aqueous two-phase system consisting of PEG 4000 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mM sodium phosphate (pH 7.00).

system, a sample was taken from the top phase for analysis in a gamma counter. The result of such a series of experiments is shown in Fig. 5. A linear relationship was found between the amount of labelled IgG bound to the cells, measured as counts transported across the interface, and the amount of ^{125}I -labelled IgG added. Thus, the amount of M-PEG-modified cells is sufficient for the amount of labelled IgG added, since no signs of saturation appeared under the experimental conditions used. These conditions were then used in a competitive assay between modified and native *Staphylococcus aureus* cells for ^{125}I -labelled IgG. The results obtained are shown in Fig. 6. As seen from this figure, cells in the region of 10^5 – 10^7 were measured.

The results clearly show that the PALA-technique can accelerate and facilitate microbiological assays. The time needed for phase separation to take place depends largely on the phase constituents used. When PEG/Dextran is used it takes approximately 1 h, whereas when PEG/ MgSO_4 is used separation is complete within 10 min. This makes the total time for one assay 40–90 min. However, still much work remains to be done before a useful method can be developed and applied in practice.

It should be pointed out that the system studied is a model system, and

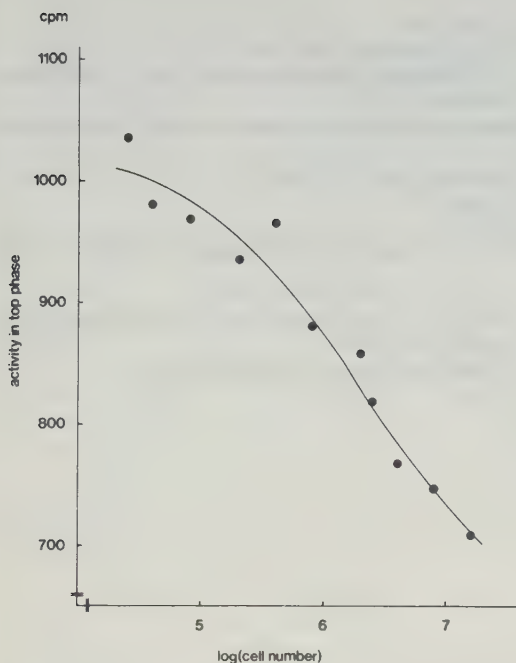


Fig. 6. Calibration curve obtained for *Staphylococcus aureus* cells obtained in a competitive binding assay, using a constant amount of ^{125}I -labelled IgG molecules, 2.6×10^{-14} mol. The diagram shows activity in the top phase as a function of number of native *Staphylococcus aureus* cells added to 6.3×10^5 M-PEG-modified *Staphylococcus aureus* cells. The mixture was incubated with ^{125}I -labelled IgG for 30 min and thereafter allowed to separate in an aqueous two-phase system consisting of PEG 6000 and Dextran T 250. A 200 μl sample was taken from the top phase for measurement of activity.

that we have taken advantage of the fact that protein A on the *Staphylococcus aureus* cell surface specifically binds IgG molecules. Work is now in progress with other systems involving antigen-antibody interactions as well as use of enzyme-labelled reagents.

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REFERENCES

- Abuchowski, A., T. Van Es, N.C. Palczuk and F. Davis, 1976, *J. Biol. Chem.* 252, 3578.
 Albertsson, P.-Å., 1971, *Partition of Cell Particles and Macromolecules*, 2nd Edn. (Almqvist and Wiksell, Uppsala).

- Albertsson, P.-Å., 1978, *J. Chromatogr.* 159, 111.
- Danielsson, D. and G. Kronvall, 1974, *Appl. Microbiol.*, 27, 368.
- Eriksson, E. and G. Johansson, 1979, in: *Methodological Surveys (B)*, Vol. 8, Cell Populations, ed. E. Reis (Ellis Horwood, Chichester) p. 81.
- Hildebrand, R.L., 1979, in: *Rapid Diagnosis in Infectious Disease*, ed. M.W. Rytel (CRC Press, Boca Raton, FL) pp. 77-88.
- Kronvall, G. and D. Frommel, 1970, *Immunochemistry* 7, 124.
- Mattiasson, B., 1980a, *J. Immunol. Methods*, 35, 137.
- Mattiasson, B., 1980b, *Anal. Lett.* 13, 851.
- Mattiasson, B. and C. Borrebaeck, 1980, in: *Enzyme Immunoassay*, ed. E.T. Magio (CRC Press, Boca Raton, FL) p. 231.
- Mattiasson, B. and T.G.I. Ling, 1980, *J. Immunol. Methods* 38, 217.
- Overby, L.R. and I.K. Muskahwar, 1979, in: *Rapid Diagnosis in Infectious Disease*, ed. M.W. Rytel (CRC Press, Boca Raton, FL) pp. 33-69.
- Thorell, J.I. and B.G. Johansson, 1971, *Biochim. Biophys. Acta* 251, 363.
- Wisdom, G.B., 1976, *Clin. Chem.* 22, 1243.

IV

Immunological Quantitation of Bacterial Cells Using a Partition Affinity Ligand Assay: A Model Study on the Quantitation of Streptococci B

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A new immunoassay for bacterial cells is described. Aqueous two-phase systems were used to separate the different reactants after binding, the principle being that in the direct assay free and bound reactants shall be recovered from different phases. In the competitive binding assay the bacteria are present in two forms—one native (to be quantified) and one modified (in fixed amount)—so that after binding and separation the complexes are recovered from different phases. ¹²⁵I-Labeled antibodies were used in the competitive assay and enzyme-labeled antibodies in the direct binding assay. In the system studied here streptococci were quantified. The direct enzyme immunoassay was more sensitive than the competitive radioimmunoassay. The smallest number of cells that could be quantified was 2500. Total time for one assay was 40-120 min.

In recent years immunochemical methods have proved useful in the analysis of bacteria. Many new immunological assays have been developed using the concepts of radio- and enzyme-immunoassay (1). Most of these assays detect low-molecular-weight antigens. The development of immunoassays for intact bacteria has, however, been impeded by difficulties in separating free from bound reactants, since such assays require a separation method that can handle both whole cells and proteins.

Partitioning in aqueous two-phase systems has recently proved useful in the separation step in binding assays of low-molecular-weight ligands and macromolecules (2,3) as well as of whole cells (4). This method has been called partition affinity ligand assay (PALA). In an assay of cells, free and bound labeled antibodies are separated in a two-phase system after the binding step, and the amount of label in one of the phases is determined. A prerequisite for an immunoassay of cells in a two-phase system is that the cells to be quantified and free antibodies are partitioned differently so that antibodies not

bound by the cells to be quantified are found in one phase and the bound ones in the other. In some cases it was possible to design a phase system in which the free and bound labeled antibodies partitioned spontaneously to different phases. A more general approach was to modify one of the reactants in order to make virtually all of it partition to one of the phases and thereby improve the separation considerably. The modification was effected by covalent attachment of one of the polymers used in the phase system.

Streptococci of type B1 were chosen for this study as they are ubiquitous and responsible for infections with a wide variety of clinical manifestations (5). There is thus a need for quick and reliable analytical methods, especially in cases where an infection may become a serious complication and even threaten the life of the patient (6).

MATERIALS AND METHODS

Materials. QAE-Sephadex A-50, Sephadex G-200, PD-10 columns (containing Sephadex G-25), and Dextran T250

were obtained from Pharmacia Fine Chemicals AB, Uppsala. Triazine and poly(ethylene glycol) with a molecular weight of 6000 came from British Drug House, Poole. Monomethoxy-poly(ethylene glycol) (MPEG,¹ trade name Carbowax) with a molecular weight of 5000 was a generous gift from Union Carbide, New York. Triethanolamine-HCl was obtained from Fluka AG, Buchs, and 4-aminoantipyrine and horseradish peroxidase (type VI) from Sigma Chemical Company, St. Louis, Missouri. Antiserum against *Streptococcus* B1 came from BBL, Division of Becton, Dickinson and Company, Cockeysville, Maryland, and from Lee Laboratories, Inc., Grayson, Georgia. All other chemicals used were of analytical grade.

Streptococcus B1 cells, heat treated and stabilized with formaldehyde, were a generous gift from Pharmacia Diagnostics AB, Uppsala. Cells were counted in a Bürker chamber using a phase-contrast microscope ($\times 400$). Attempts were made to count individual cells.

Modification of streptococci with monomethoxy-poly(ethylene glycol). MPEG was activated with triazine (7). In the coupling step activated MPEG was added to a suspension of streptococci (1.2×10^8 cells/ml) with 75 mmol/liter triethanolamine-HCl, pH 9.4. Unless otherwise stated, 10 mg activated MPEG was added per milliliter of suspension. After agitation for 1 h at room temperature the cells were stored at 4°C.

Purification of antibodies. Antibodies were purified from whole serum by ion-exchange chromatography on a QAE-Sephadex A-50 column equilibrated with sodium phosphate buffer, pH 7.00, ionic strength 0.10 mol/liter. The fraction that was not adsorbed to the ion exchanger was collected and concentrated.

¹²⁵I-labeled antibodies. Antibodies were prepared with the lactoperoxidase method

(8). One millicurie of ¹²⁵I was added to 0.85 mg of antibodies in 0.04 mol/liter sodium phosphate buffer at pH 7.00 and incubated for 10 s at room temperature. Free ¹²⁵I was separated from the antibodies in a PD-10 column. The yield in the labeling step was 90%.

Enzyme-labeled antibodies. Antibodies were prepared with the sodium *meta*-periodate method (9). One milligram of horseradish peroxidase was activated and mixed with 2 mg antibody. The conjugate formed was separated from unreacted protein on a Sephadex G-200 column (1.6×72 cm).

Competitive binding assay. In the competitive binding assay streptococci in a sample were mixed with a fixed amount of MPEG-modified streptococci and allowed to compete for a limited amount of ¹²⁵I-labeled antibody. The total volume was 100 μ l and contained 0.1 mol/liter sodium phosphate buffer, pH 7.00. The reagents were mixed and incubated at room temperature for 30 or 60 min and then separated as described below.

Direct binding assay. In the direct binding assay streptococci in a sample were mixed with a fixed amount of antibody labeled with peroxidase in a total volume of 100 μ l. The buffer used consisted of 0.010 mol/liter Tris-HCl, 0.090 mol/liter KCl, pH 7.00. The buffer and the phase system components were autoclaved before use. After partitioning as described below a 200- μ l sample was taken from the top phase and mixed with 800 μ l reagent solution, giving a final concentration of 14 mmol/liter phenol, 0.8 mmol/liter 4-aminoantipyrine, 1 mmol/liter hydrogen peroxide, and 10 mmol/liter Tris-HCl, pH 7.00. The enzyme activity was measured in a spectrophotometer at 510 nm.

Separation using a two-phase system. In a typical assay 900 μ l of a premixed phase system consisting of 5% (w/w) PEG-6000 and 7.5% (w/w) Dextran T250 was added to 100 μ l of incubation mixture and thoroughly mixed on a Vortex-type mixer. The mixture was left for 60 min at room tem-

¹ Abbreviation used: MPEG, monomethoxy-poly(ethylene glycol).

perature to allow the phase separation to take place. Two distinct phases were formed and the free and bound antibodies were separated to different phases. A 200- μ l aliquot was taken from the top phase (or from each phase) and the amount of labeled antibody was determined. The distribution of antibodies is expressed by the partition coefficient, defined as the ratio between the concentrations in the top and bottom phases, respectively: $K_{\text{part}} = C_{\text{top}}/C_{\text{bottom}}$.

RESULTS AND DISCUSSION

Bacterial cells were quantified with both competitive and direct binding assays. The latter assay is simpler but requires that the cells and the antibodies partition to different phases. However, when studying the spontaneous partitioning of the native reactants in an aqueous two-phase system, it was found in the case of streptococci that the cells and the antibodies both partition to the bottom phase and that no effective separation of the reactants could be achieved, not even when the ionic composition of the phase system was varied. In such a situation it is possible to change the partitioning by modification of the surface properties of either of the two reactants in the binding assay (3,4). The species (here streptococci B cells) to be quantified is for practical reasons not suitable for chemical treatment. Furthermore, modification of the antibodies may lead to deactivation. A search was therefore made for other solutions.

In the competitive binding assay (Fig. 1), varying amounts of streptococci from a sample and a fixed amount of MPEG-modified streptococci are incubated with a limiting amount of labeled antibody. The incubation mixture is then partitioned in an aqueous two-phase system. The modification aims at getting the two bacterial reactants to partition to different phases in the separation step. In the phase system used, consisting of PEG and Dextran, unmodified streptococci partition mainly to the Dextran-rich bottom

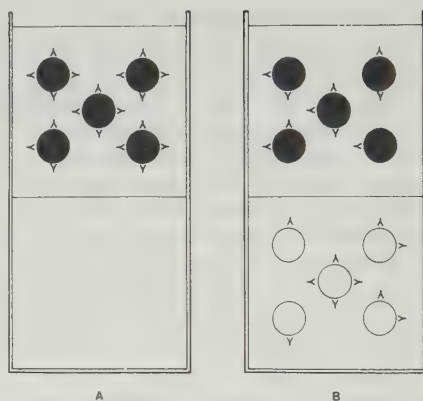


FIG. 1. Schematic representation of the competitive assay. Unmodified bacteria from the sample and PEG-modified bacteria are mixed with 125 I-labeled antibodies and incubation for 60 min. The phase system is added, and after 60 min for separation the radioactivity in one or both of the phases is determined. (A) MPEG-modified streptococci (●) partition to the top phase and thereby transport bound antibodies (Y). (B) When unmodified streptococci (○) are present they compete with the MPEG-modified streptococci for the antibodies. Since the unmodified cells partition to the bottom phase the result is a decrease in the activity in the top phase.

phase. The modified cells should then be found in the PEG-rich top phase, so that a competitive situation can be exploited in the binding step and, in the subsequent partitioning step, the distribution of the bound labeled antibody will reflect the relative amounts of unmodified and modified cells, respectively.

In an assay for *Staphylococcus aureus* (4), the cells were made to partition almost exclusively to the top phase by covalent attachment of MPEG. It was found that there is an optimum degree of modification achievable by variation of the amount of MPEG molecules added per cell or biomolecule. At a low degree of modification the surface properties are not changed enough to bring about satisfactory partitioning. On the other hand, when too many MPEG molecules are attached they may be a steric hindrance to the binding of antibodies to the antigenic determinants. For the streptococci an opti-

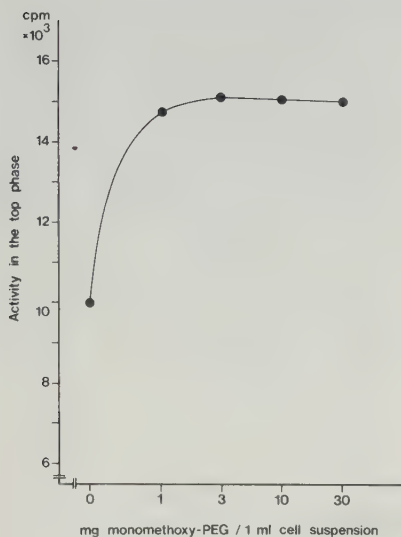


FIG. 2. Determination of the capacity of MPEG-modified streptococci to transport antibodies to the top phase for different degrees of modification using the conditions described for the competitive assay. The activity in the top phase is shown as a function of the amount of activated MPEG added per milliliter of reaction mixture (1.2×10^8 cells/ml).

imum degree of MPEG modification was obtained with 3 mg activated MPEG added per 10^8 cells, as determined by the partitioning of bound ^{125}I -labeled antibodies (Fig. 2). The partitioning was not significantly altered by a 10-fold larger amount of MPEG, which allowed a high degree of modification without significant loss of transport capacity under the conditions used. In all subsequent experiments a modification of 10 mg MPEG per 10^8 cells was used. Under the experimental conditions used, the partitioning of ^{125}I -labeled antibodies bound to the modified streptococci did not vary with the amount of antibody added; i.e., the bound antibodies did not markedly affect the partitioning of the cells. The amount of labeled antibody found in a phase was therefore taken as a function of the number of cells in that phase.

When designing a competitive binding

assay it is important to select the proper amounts of reagents that are to be used in the assay. If a fixed amount of ^{125}I -labeled antibodies is used the number of MPEG-modified cells necessary to transport the antibodies to the top phase has to be determined (Fig. 3).

The conditions for the partitioning were chosen in such a way that unmodified streptococci partitioned to the bottom phase. The partitioning of the PEG-modified cells was rather insensitive to variation of the composition of the phase system. It was thus possible to optimize the partitioning so that one of the two bacterial entities partitioned completely to one phase, and the other to the other phase. The partitioning of the labeled antibodies was, however, not critical since the sites on the bacterial cells were present in heavy excess. The specific antibodies could therefore be assumed to be bound to the cells. Thus, the partitioning of the label is a function of the ratio between the number of modified and the number of unmodified

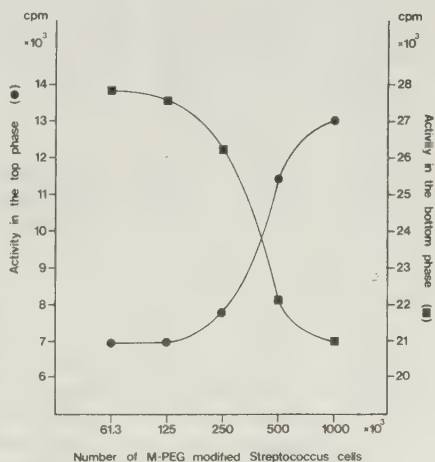


FIG. 3. Determination of the proper amount of MPEG-modified bacteria needed to transport antibodies to the top phase. The degree of modification used was 10 mg activated MPEG/ml. The conditions described for the competitive assay were used.

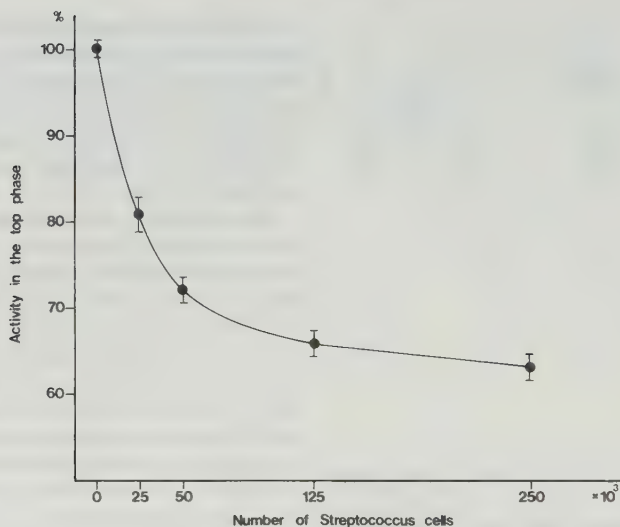


FIG. 4. Standard curve for the competitive assay of streptococci under the experimental conditions described in the text. The number of MPEG-modified streptococci present was 30,000. On the activity scale 0% denotes the activity with no cells in the system and 100% the activity with only MPEG-modified cells present.

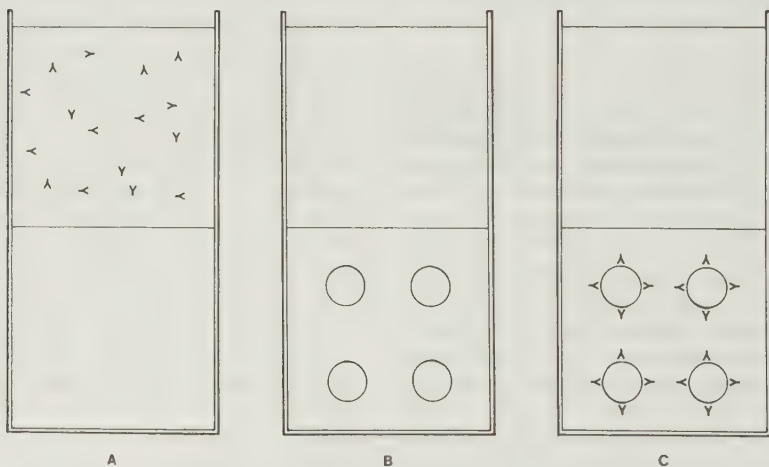


FIG. 5. Schematic representation of the direct binding assay. Unmodified bacteria from the sample and enzyme-labeled antibodies are mixed and incubated for 60 min. The phase system is added and after another 60 min for separation the enzymatic activity in the top phase is determined. (A) The enzyme-antibody conjugate (Y) partitions mainly to the top phase. (B) The unmodified streptococci (O) partition to the bottom phase. (C) When the enzyme-labeled antibodies are bound to the bacteria, they are transported to the bottom phase with a consequent decrease in the enzyme activity of the top phase.

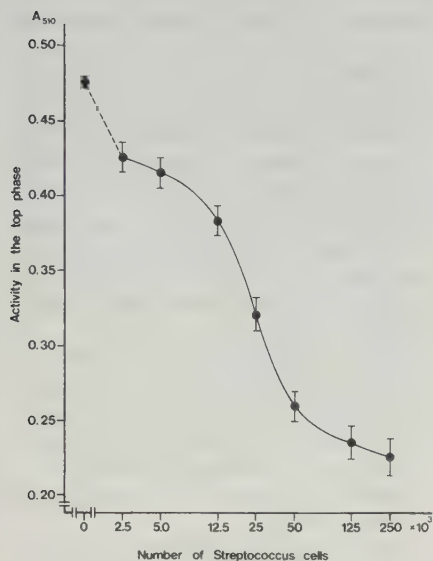


FIG. 6. Standard curve for the direct binding assay of streptococci using the experimental conditions described in the text. A sample from the top phase is drawn and added to a solution containing substrates for the enzyme. The absorbance is read after a predetermined time, usually 1 h.

bacteria, respectively. A standard curve for the quantitation of streptococci was obtained (Fig. 4).

The whole immunoglobulin G fraction of the antiserum was labeled and used in the assay, with the result that there was a large amount of antibody without specificity for the streptococci present. Consequently, there was a high background activity (Fig. 3), which limited the sensitivity of the assay. This limitation can, if necessary, be overcome by further purification of the antibodies with, e.g., affinity chromatography. Under the experimental conditions used, the sensitivity of this competitive assay depends mainly on the competitive situation per se, since the smallest detectable amount of streptococci in the assay cannot be much smaller than that of the MPEG-modified cells used.

The limiting factors of a competitive sys-

tem can be obviated by the use of a direct binding assay in which the binding of antibodies to cells is measured (Fig. 5). A prerequisite is that the two reactants partition differently in a two-phase system so that the bacteria can transport bound antibodies across the phase boundary. In the direct binding assay illustrated in Fig. 5, the antibodies were labeled with an enzyme, giving an enzyme-antibody conjugate with an appropriate partition coefficient so that further modification was unnecessary. The partitioning of the reactants was optimized by replacing the sodium phosphate buffer used in the competitive assay with a buffer consisting of Tris/KCl. The standard curve for the direct assay is shown in Fig. 6, from which it is clear that the sensitivity was significantly better than that of the competitive assay.

The partitioning of molecules or cells in a two-phase system is not sensitive to other molecules or cells present in the system. The separation is thus not influenced by, e.g., serum or cell homogenate, as long as the ionic strength and pH of the system are not changed. The PALA procedure has been used for determination of triiodothyronine in serum with about the same precision and accuracy as obtained with commercial kits (10).

A complication in partitioning cells is the possibility of adsorption of cells to the interface. Cells may be distributed between one of the two bulk phases and the interface (11), but the adsorption can often be kept at a low level by the use of a suitable phase system. With the PEG-Dextran system used, it was found that on addition of MPEG-modified streptococci the decrease in activity in one phase was balanced by an increase in the other (Fig. 3), indicating that the adsorption to the interface did not affect the outcome of the assay.

When quantifying cells with immunological procedures not only viable cells are detected, but also dead cells and cell fragments with the antigenic determinant. Therefore,

an immunoassay may give a higher number of cells than what is obtained with counting procedures. In a study where yeast cells (*Saccharomyces cerevisiae*) were quantified by the binding of radiolabeled concanavalin A to the cell surface (12), it was found that the PALA procedure gave about 5% higher values than those obtained by counting in a Bürker chamber.

Identification of streptococci has conventionally been based on the extraction of group-specific surface carbohydrate antigens (13) and subsequent identification with immunological methods. In order to compensate for the low sensitivity in immunoprecipitation methods the bacteria must be cultured overnight before analysis (14). Recently, a more sensitive immunoassay has been applied in the grouping of whole streptococci (15), but the cells must still be cultured overnight.

The present method is quick (40–120 min) and sensitive. In the development of new analytical techniques for bacterial cells it is desirable to try to design assays sensitive enough to make preculture of the cells unnecessary.

REFERENCES

1. Rytel, M. W. (1979) in *Rapid Diagnosis in Infectious Disease* (Rytel, M. W., ed.), pp. 7–14, CRC Press, Boca Raton, Fla.
2. Mattiasson, B. (1980) *J. Immunol. Methods* **35**, 137–146.
3. Mattiasson, B., and Ling, T. G. I. (1980) *J. Immunol. Methods* **38**, 217–223.
4. Mattiasson, B., Ling, T. G. I., and Ramstorp, M. (1981) *J. Immunol. Methods* **41**, 105–114.
5. Jelinkova, J., Neubauer, M., and Duber, J. (1970) *Zentralbl. Bakteriол. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig.* **214**, 450–457.
6. Quintiliani, R., and Engh, G. A. (1971) *J. Bone Joint Surg.* **53**, 1391–1399.
7. Abuchowski, A., van Es, T., Palczuk, N. C., and Davies, F. F. (1977) *J. Biol. Chem.* **252**, 3578–3581.
8. Thorell, J. I., and Johansson, B. G. (1971) *Biochim. Biophys. Acta* **251**, 363–369.
9. Wilson, M. B., and Nakane, P. K. (1978) in *Immunofluorescence and Related Staining Techniques* (Knapp, W., Holubar, K., and Wick, G., eds.), pp. 215–224, Elsevier, Amsterdam.
10. Mattiasson, B., and Eriksson, H. (1982) *Clin. Chem.*, in press.
11. Albertsson, P.-Å. (1971) *Partition of Cells, Particles and Macromolecules*, 2nd ed., p. 138, Almqvist & Wiksell, Uppsala.
12. Mattiasson, B., Ling, T. G. I., Nilsson, J., and Dürholt, M. (1982) *Lectins—Biology, Biochemistry, Clinical Biochemistry*, Vol. 2, de Gruyter, Berlin, in press.
13. Lancefield, R. C. (1938) *Proc. Soc. Exp. Biol. Med.* **38**, 473–478.
14. Chitwood, L. A., Jennings, M. B., and Riley, H. D. (1969) *Appl. Microbiol.* **18**, 193–197.
15. Cumming, C. G., Ross, P. W., Poxton, I. R., and McBride, W. H. (1980) *J. Med. Microbiol.* **13**, 459–461.

V

ULTRAFILTRATION AFFINITY PURIFICATION

ISOLATION OF CONCAVALIN A
FROM SEEDS OF CANAVALLIA ENSIFORMIS

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A new approach to affinity purification is described here in which the combination of affinity binding with ultrafiltration separation creates an efficient purification method.

In this approach, a macromolecular ligand is retained on one side of a membrane and is allowed to interact with a crude extract. Material specifically binding to the ligand forms a high molecular weight complex whereas all other material is washed out through the membrane pores. Upon addition of free ligands or other dissociation media the complexes are split and the formerly bound material is liberated, passed through the membrane and collected in a purified state.

The system described in this paper is the purification of Concanavalin A from a crude extract of Canavalia ensiformis using the surface structures of heat killed yeast cells (Saccharomyces cerevisiae) as macromolecular ligands.

High capacity for Con A was demonstrated. The yield over the total process was approximately 70 % and the product was homogeneous when analyzed by SDS-polyacrylamide gel electrophoresis.

The introduction of affinity chromatography has revolutionized protein purification from a laborious trial and error procedure into one with high resolution, efficiency and relative operational simplicity. In order to set up an affinity chromatographic purification step, a ligand must first be available that can be coupled to a solid matrix. Chemical modification to introduce appropriate groups on the ligands is the limiting step when setting up an affinity purification step today.

Even if affinity techniques are routinely used in many laboratories, rather few new technical developments have been presented recently, and thus affinity purification technology is in essence the same today as ten years ago.

In modern biotechnology, the ability to produce molecules of unique structures and specific biological activities in large scale has focused the attention to the weakest point in the total process - the down stream processing step. Some efforts have been done to improve capacity of affinity chromatographic procedures (1) in order to solve this problem, but so far no solution that works sufficiently well has been presented.

In parallel with the development in affinity chromatography, membrane technology has grown into an established technique for fractionation of molecules based on their molecular size (2).

The present report describes the use of ultrafiltration technology in combination with affinity purification technology, thereby generating a purification process with great potential for use in large scale and in continuous processes.

CHEMICALS USED

Yeast cells, Saccharomyces cerevisiae, were obtained from a local source. Finely ground, defatted Jack bean meal and horse radish peroxidase (E.C.1.11.1.7), type II and 2,2'-azino-di-(3-ethylbenz thiazoline sulfonic acid) (ABTS) were purchased from Sigma Chemical Company, St. Louis, USA. Anti-Concanavalin A antiserum was from E-Y-Laboratories Inc., San Mateo, CA, USA. All other chemicals were of analytical grade.

The hollow fiber units, pumps, level monitors and pressure meters were generously supplied by Gambro AB, Lund, Sweden.

PREPARATION OF CRUDE EXTRACT
OF JACK BEANS (Canavalia ensiformis)

Five hundred grams of defatted, ground Jack beans (Canavalia ensiformis) were suspended in 2.5 L 0.9 % NaCl, containing 1 mM each of Ca^{2+} , Mg^{2+} and Mn^{2+} , pH 7.0. The suspension was stirred overnight at 4 °C and then filtered through cheese cloth. The filtrate was stored at 4 °C, and the extraction procedure was repeated once on the solid residue. The two filtrates thus obtained were pooled and centrifuged for 1 hour at 14,500 x g at 4 °C. The supernatant containing the lectin was stored at 4 °C.

PREPARATION OF AFFINITY SORBENT MATERIAL

Cells of Saccharomyces cerevisiae were used as affinity sorption material. They were heat killed before use.

150 grams of yeast cells, pressed weight, were suspended in 1.0 L 0.9 % NaCl solution (9 grams /L). The cell suspension was continuously stirred for 40 minutes in a water bath at 70 °C.

Soluble impurities in the cell suspension were removed at continuously by at room temperature recirculating the cells suspension through a hollow fiber unit with a molecular weight cut-off of 1.000.000 D. NaCl-solution (9 grams/L) was continuously added to keep the volume constant. Washing proceeded until the effluent from the outlet of the membrane unit showed no absorbance at 280 nm.

QUANTITATIVE DETERMINATION OF CONCAVALIN A

To quantitate Con A in the various samples, an enzyme linked immunosorbent assay (ELISA) was set up. The assay was performed in Dynatechs Microelisa microtiter plates, with flat bottomed wells, which are easily monitored in multiscanning photometers.

The wells were coated by pipetting 100 μ L of a coating solution containing 5 μ grams rabbit anti-Concanavalin A antibodies per 1 mL of 0.1 M carbonate buffer, pH 9.5 per well. Incubation proceeded for 2 hours at 37 $^{\circ}$ C in a moisture chamber. The wells were then thoroughly washed with a solution containing 0.9 % NaCl and 0.05 % Tween 20.

The samples to be assayed for Con A were prepared by dilution with a standard dilution buffer, comprised of 0.1 M sodium phosphate and 0.05 % Tween 20, pH 8.0. One hundred μ L of diluted sample was added to each well. Plates were subsequently incubated and washed as described above. Con A present in the sample was bound by the anti-Concanavalin A antibodies coupled to the wells. The amount of Con A bound to the anti-Concanavalin A antibodies is directly proportional to the amount of Con A present in the incubation mixture.

To visualize Con A in the wells, incubation with 100 μ L of a horse radish peroxidase solution (40 μ grams per 1 mL of standard dilution buffer) was performed as in the previous step. Horse radish peroxidase, a glycoprotein, interacts with Con A, through a lectin-carbohydrate interaction.

The amount of peroxidase bound was then determined by incubation with a substrate solution composed of 20 mL 50 mM citrate buffer, pH 4.0, 5.33 μ L 30 % hydrogen peroxide and 100 μ L 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (439 mg dissolved in 20 mL distilled water). 200 μ L of this substrate solution was incubated per well for 15 minutes, after which the absorbance at 405 nm was read in a photometer (Titertek Multiscan).

BIOASSAY OF CONCAVALIN A

The biological activity of Concanavalin A in the various samples was determined by its capability to agglutinate human red blood cells. The hemagglutination test was performed in Titertechs Microtiter plates, with v-shaped wells.

Each sample was diluted in serial two-fold dilutions. One hundred μ L of diluted sample and 100 μ L of a 2 % red blood cell suspension were added per well. The plate was incubated for 1 hour at room temperature. The plate was then visually inspected for estimation of the agglutination endpoint of each dilution series.

THE AFFINITY PURIFICATION PROCESS FOR CONCAVALIN A

The procedure discussed here for the purification of Concanavalin A from a crude extract is based on the fact that Con A interacts with carbohydrate residues exposed on the surface of Saccharomyces cerevisiae cells. The experimental set-up is schematically illustrated in Figure 1. The total system is built up around a hollow fiber unit with a molecular weight cut-off of 1.000.000 D. The hollow fiber unit is connected to a peristaltic pump (A), which continuously recirculates a stream containing the affinity sorbent material (i.e. the yeast cells) mixed with the material to be purified, through the inner parts of the fibers (flow rate 500 mL/minute). Material in the recirculation stream which is

capable of penetrating the membrane pores leaves the interior of the hollow fiber (containing the cells) and enters the outer parts of the membrane unit. The outlet from the outer shell of the membrane unit is connected to a photometer for the continuous monitoring of the absorbance at 280 nm in the effluent. By monitoring the effluent from the membrane unit, the purification process is conveniently followed.

The recirculation pump (A) is connected to a pressure meter (B), placed between the outlet of the pump and the inlet of the membrane unit. The recirculation pump is controlled by the pressure in this stream. This security system is used in order to protect the hollow fiber membrane from exposure to harmful pressures. The level of liquid in the beaker (C) is controlled by a level sensor controlling a pump (D).

The subsequent washing and dissociation steps were performed using a similar experimental set-up.

After all non-interacting material has been washed out of the system, the only material present in the recirculation stream are the cells with bound Con A. Dissociation of this complex is performed by addition of glucose (150 grams/L), which competes with the sugar residues on the cell surface for binding of Con A. The complex of Con A-glucose has a molecular weight below the cut-off of the membrane, and thus leaves the system.

The dissociation process is continued until the absorbance at 280 nm in the effluent has regained a value of zero. The affinity sorbent material is then regenerated by addition of an appropriate buffer.

To study the purity of the fractions containing Concanavalin A, SDS-polyacrylamide gel electrophoresis was performed. The 80 ugram samples were applied to a 15 % polyacrylamide gel, prepared according to Davis (3). Electrophoresis was performed for 1 hour at 15 mA and 3 hours at 30 mA, pH 8.3.

QUANTITATION OF CONCAVALIN A

In order to monitor the purification procedure, assays for the purified substance had to be set up. An ELISA-procedure using rabbit anti-Concanavalin A antibodies were used. The bound Concanavalin A was visualized by addition of the glycoprotein, horse radish peroxidase, that form coloured products upon catalysis. A generalized presentation is shown in Figure 2.

Using the microtiter plate ELISA-method for quantitation of Con A, a calibration curve as shown in Figure 3 was obtained. The biological activity, expressed as red blood cell clotting activity was also tested.

BATCH-USE ULTRAFILTRATION AFFINITY PURIFICATION

When using this concept of ultrafiltration affinity purification, elution profiles like that illustrated in Figure 4 are obtained. As stated earlier, the principle behind ultrafiltration affinity purification is to use membranes with a pore size distribution, sufficient to let individual substances in the extract pass freely through the membrane, but at the same time retaining the substance of interest by interaction with a macromolecular ligand which is large enough to be included in the unit.

Material with a molecular weight below the cut-off limit is forced through the membrane pores and leaves the recirculation stream containing ligand. This flow of liquid is passed through a photometer to continuously monitoring its absorbance at 280 nm.

When all impurities are washed out and a stable base line is obtained from the photometer, the elution may be started. This is done by introducing a dissociating medium into the recirculation stream. This medium should contain molecules which will compete with the ligand residues for the binding of the substance of interest. New complexes will thus be formed, with a molecular weight below the cut-off of the membrane pores, allowing the complex to leave the recirculation stream containing the macromolecular ligand. The second peak monitored at 280 nm in the effluent is due to material thus leaving the membrane unit.

The eluted material is collected in a large vessel. As well, fractions are also collected during the entire elution process for later examination with the analytical tests described earlier. The eluate is dialysed in a similar experimental set-up using a hollow fiber unit with a cut-off of 1.000 D. This unit allows removal of dissociating ligand (i.e. glucose) from the substance of interest (Con A), with simultaneous concentration of this final product.

In our process 10 mL fractions, which were collected during both elution processes, were assayed with respect to the immunological reactivity as well as the biological activity of the Con A.

Figure 4. shows the recordings from the photometer (solid line) during the above mentioned experiment. Two peaks were obtained. The first of these peaks represents the non-interacting material and the second peak, material eluted from the cells upon glucose addition.

The results from the ELISA-tests of the fractions are represented in the figure (dotted line). The first peak was shown to contain some immunological reactive Concanavalin A whereas the major part of the Con A was detected in the second peak. The first peak, however, gave no observable agglutination of red blood cells (line with squares). Thus, these Con A molecules not interacting with the cells are immunologically detectable whereas they are not able to agglutinate red blood cells. The second peak, representing the glucose eluted material, was capable of agglutinating red blood cells.

In an experiment lasting for a total of 5 hours, 3.4 grams of Con A were purified. The results from SDS-polyacrylamide

gel electrophoresis on the purified material are shown in Figure 5. The Con A preparation was shown to contain one single band when analyzed with gel electrophoresis. The yield in the total purification process was 70 %.

DISCUSSION

The technique as described here is based upon the same inherent properties of the binding protein as those utilized in conventional affinity chromatographic procedure. The unique features with the technique discussed here is that the different unit operations - application, washing, elution and reconditioning - can take place at different locations in the room, whereas in conventional procedures all steps are performed consecutively in the same column.

By using membrane technology it is possible to design a system where the ligand is transported between different membrane units where different unit operations take place. It will then be possible to operate such a process continuously, with the ligand kept and recircled in a closed volume. The steps demonstrated in this report indicate that each step operates smoothly individually. Attempts to operate the whole reaction sequence in an integrated manner are currently in progress in our laboratory, and are not discussed here.

When setting up an ultrafiltration affinity purification system, it is very important to use proper membranes This

was initially a limiting factor in our efforts since we operated with pore sizes large enough to let a protein of mwt of 10^6 pass through, and such membranes are at present rarely available on the market. The ligand used had to be either coupled to high molecular weight dextran (mwt 2.000.000) or, as in the case studied here, to small particles (dead yeast cells).

The total molecular weight of the macromolecular ligand must be such that it shows no tendency to leak from the membrane units. It is thus recommendable that before coupling a ligand to the macromolecular structure, the latter is extensively prefractionated in a membrane unit to eliminate all low molecular weight material. The same approach holds when small particles, e.g. cells, are used as supports. In those cases fragments of cell surface structures may ruin the purification process. To minimize these risks, dead cells and even cells treated with crosslinking reagents may be used.

In order to operate an ultrafiltration affinity purification process efficiently, high demand must be made on the characteristics of the membranes used. Well defined molecular weight cut-offs are a must for the membrane used in the binding, washing and the elution steps, whereas in the reconditioning steps membrane units with a low molecular weight cut-off can be used.

Important criteria for a new purification process are purity and yield of the product. In the system described here, purity of the obtained Con A was tested with SDS-polyacrylamide gel electrophoresis and was found to be high. One single band was obtained. Yield in the overall process was 70 %.

In conclusion: Ultrafiltration affinity purification is a powerful purification technique based on combining affinity interactions in free solution with membrane technology. The technique promises to be useful in processes which are operated in a continuous manner and may thus be useful for large scale purification.

LEGENDS TO FIGURES

Figure 1. A schematic representation of the experimental set-up used for the purification of Concanavalin A from a crude extract of Canavalia ensiformis. The diagram illustrates an ultrafiltration membrane system consisting of a hollow fiber unit with a molecular weight cut-off of 10^6 , (long column), a peristaltic pump (A) for the continuous recirculation of the affinity sorbents, a level monitor placed on the outside of (C) a beaker. The level monitor is connected to a second peristaltic pump (D), which keeps the volume of the recirculation stream at a constant, predetermined value.

Figure 2. The principle of the enzyme linked immunosorbent assay (ELISA) used in the quantitation of Concanavalin A.

A.

The wells of a microtiterplate were coated with anti-Concanavalin A antibodies.

B.

The antibody coated wells were incubated with samples containing Con A. Con A will interact with anti-Concanavalin A antibodies.

C.

Peroxidase was then incubated in the wells. The glycoprotein peroxidase will interact with Con A, bound to the anti-Concanavalin A antibodies.

Figure 2 Incubation of an enzyme substrate, which upon enzymatic catalysis forms a coloured product, thus giving an estimation of the amount of peroxidase which is present in the wells, as well as an indirect measure of the amount of Con A bound to the wells. This product formation can then be correlated to the amount of Con A present in the incubation mixture. Experimental details are given in the text. 19

Figure 3. A calibration curve for Concanavlin A obtained in the enzyme linked immunosorbent assay (ELISA). The experimental details are given in the text.

Figure 4. Results from an ultrafiltration affinity purification experiment. Concanavlin A, was purified in a hollow fiber membrane system using surface structures on heat killed yeast cells as adsorbent. Fractions, 10 mL, were collected once a minute.

The solid line shows the elution profile obtained when monitoring the effluent at 280 nm. The first peak results after the application of 500 mL crude extract of Canavalia ensiformis and contains non-specifically binding material. The second peak results from change from binding solution to dissociating solution containing glucose. There Con A is eluted from the affinity ligands (yeast cells).

Results from the ELISA performed on the collected fractions is shown by the dotted line ().

Results from the hemagglutination study of the collected and dialyzed fractions is given by the line containing squares.

Further experimental details are given in the text.

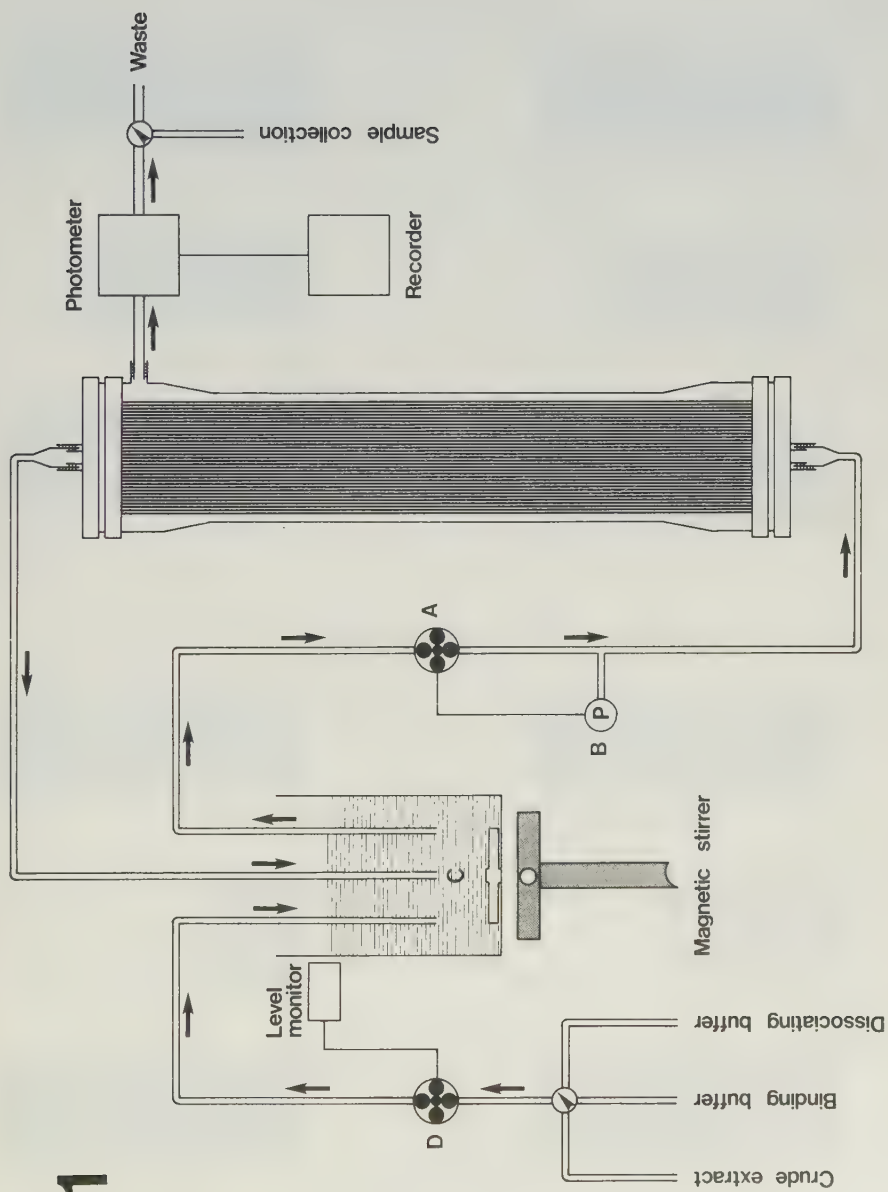
Figure 5. Results from the SDS-polyacrylamide
gelelectrophoresis.

A. The crude extract.

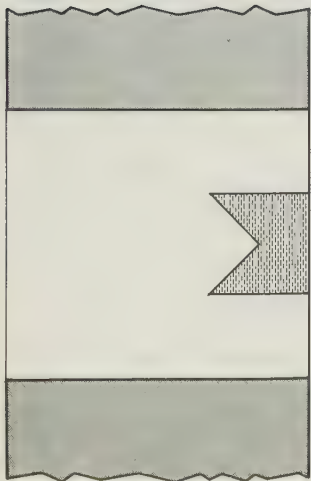
B. The purified Con A.

REFERENCES

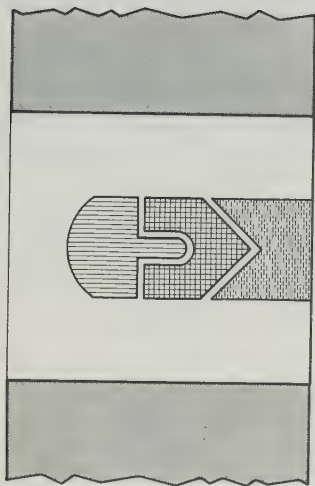
1. J.J. Hughes and S.E. Charm
"Methods for Continuous Purification of
Biological Material Using Immunosorbent"
Biotechnology and Bioengineering (1979)
21, 1439-1455
2. A.S. Michaels
"Membrane Technology and Biotechnology"
Desalination (1980)
35, 329-351
- 3 B.J. Davis (1964)
Ann. N.Y. Acad. Sci., 121, 404



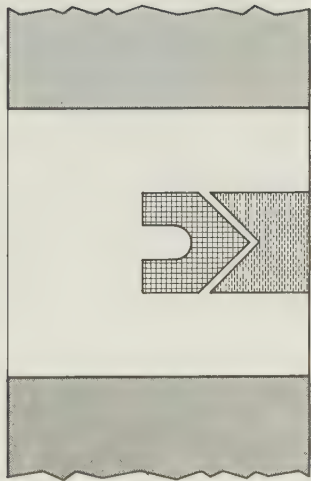
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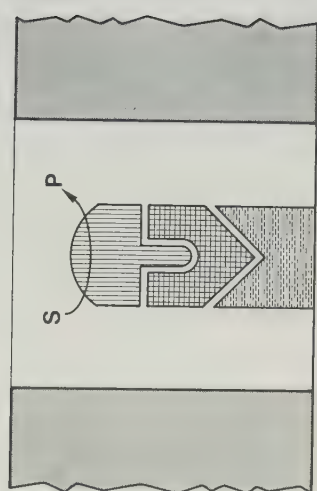
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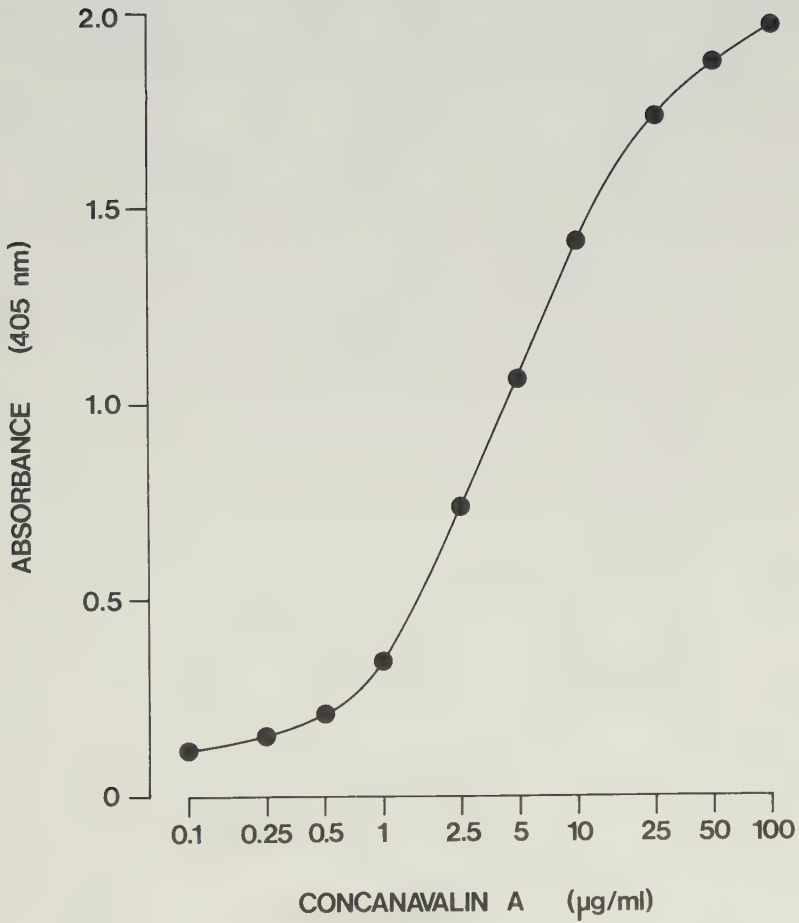
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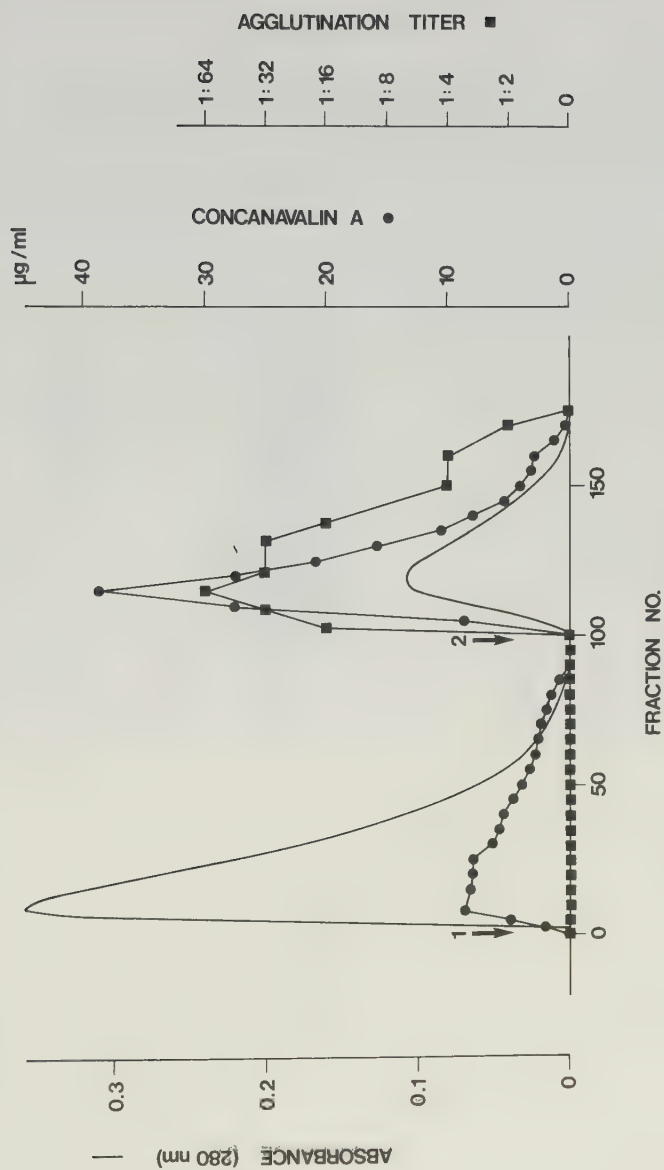


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